

Original Paper

Enhanced Erythrocyte Membrane Exposure of Phosphatidylserine Following Sorafenib Treatment: An *in vivo* and *in vitro* Study

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Key Words

Phosphatidylserine • Sorafenib • Calcium • Cell volume • Eryptosis • Oxidative stress

Abstract

Background: Sorafenib (Nexavar®), a polytyrosine kinase inhibitor, stimulates apoptosis and is thus widely used for chemotherapy in hepatocellular carcinoma (HCC). Hematological side effects of Nexavar® chemotherapy include anemia. Erythrocytes may undergo apoptosis-like suicidal death or eryptosis, which is characterized by cell shrinkage and phosphatidylserine-exposure at the cell surface. Signaling leading to eryptosis include increase in cytosolic Ca^{2+} -activity ($[\text{Ca}^{2+}]_i$), formation of ceramide, ATP-depletion and oxidative stress. The present study explored, whether sorafenib triggers eryptosis *in vitro* and *in vivo*. **Methods:** $[\text{Ca}^{2+}]_i$ was estimated from Fluo3-fluorescence, cell volume from forward scatter, phosphatidylserine-exposure from annexin-V-binding, hemolysis from hemoglobin release, ceramide with antibody binding-dependent fluorescence, cytosolic ATP with a luciferin-luciferase-based assay, and oxidative stress from 2',7' dichlorodihydrofluorescein diacetate (DCFDA) fluorescence. **Results:** A 48 h exposure of erythrocytes to sorafenib ($\geq 0.5 \mu\text{M}$) significantly increased Fluo 3 fluorescence, decreased forward scatter, increased annexin-V-binding and triggered slight hemolysis ($\geq 5 \mu\text{M}$), but did not significantly modify ceramide abundance and cytosolic ATP. Sorafenib treatment significantly enhanced DCFDA-fluorescence and the reducing agents N-acetyl-L-cysteine and tiron significantly blunted sorafenib-induced phosphatidylserine exposure. Nexavar® chemotherapy in HCC patients significantly enhanced the number of phosphatidylserine-exposing erythrocytes. **Conclusions:** The present observations disclose novel effects of sorafenib, i.e. stimulation of suicidal erythrocyte death or eryptosis, which may contribute to the pathogenesis of anemia in Nexavar®-based chemotherapy.

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Introduction

Sorafenib (Nexavar®) is a polytyrosinekinase inhibitor used in the treatment of malignancies such as hepatocellular carcinoma (HCC) and renal cell carcinoma [1-8]. The substance is further considered for the treatment of non small cell lung carcinoma [9]. Sorafenib inhibits a wide variety of kinases including several kinases of the Raf family, platelet-derived growth factor receptor, vascular endothelial growth factor receptors 1 and 2, c-Kit, and Fms-like tyrosine kinase 3 [10]. The drug is at least partially effective by inducing suicidal death of tumor cells [11-14], an effect at least partially due to Raf inhibition [10]. The induction of apoptosis by sorafenib involves activation of caspases [12, 15, 16]. Side effects of sorafenib include anemia [17]. The underlying cause of the anemia is incompletely understood. In theory, Nexavar®-induced anemia may be triggered by excessive bleeding [18, 19]. Alternatively, the mechanisms leading to Nexavar® induced death of tumor cells could similarly affect the survival of erythrocytes. Similar to apoptosis of nucleated cells, the suicidal death of erythrocytes or eryptosis eventually leads to the disposal of the affected cells [20]. Following triggering of eryptosis the clearance of circulating erythrocytes may exceed the formation of new erythrocytes thus leading to anemia [20]. The possibility that the anemia following Nexavar® treatment may at least partially be due to triggering of eryptosis, has, to the best of our knowledge, never been tested.

The present study thus explored, whether Nexavar® is indeed able to trigger eryptosis. Similar to apoptosis of nucleated cells, eryptosis is characterized by cell membrane scrambling and cell shrinkage [20]. Eryptosis can be triggered by Ca^{2+} entry through Ca^{2+} -permeable cation channels [21, 22]. An increase in the cytosolic Ca^{2+} concentration is followed by activation of Ca^{2+} -sensitive K^{+} channels [23] resulting in cell shrinkage due to K^{+} exit, hyperpolarization, Cl^{-} exit and thus cellular KCl loss together with osmotically obliged water [24]. An increased cytosolic Ca^{2+} concentration further stimulates cell membrane scrambling with phosphatidylserine exposure at the cell surface [25]. In addition, eryptosis is triggered by ceramide formation [26], energy depletion [27], caspase activation [28-32] and by modulation of the activity of protein kinases such as AMPK [22], cGKI kinase [33], JAK3 [34] CK1 [35] and p38 MAPK [36].

The present study explored, whether sorafenib (Nexavar®) stimulates eryptosis and, if so, to elucidate underlying mechanisms.

Materials and Methods

Patients, erythrocytes, solutions and chemicals

To examine the *in vivo* effects of sorafenib, EDTA blood samples from patients with a known diagnosis of hepatocellular carcinoma (HCC) were taken prior to and after administration of Nexavar® (800 mg/day; Bayer, Bergkamen, Germany) both after a single and after multiple administrations. The study is approved by the ethics committee of the University of Tübingen (184/2003V). All patients were fully informed about the aims and procedures of the study and all patients provided a statement confirming informed consent.

To study the *in vitro* effects of sorafenib, leukocyte-depleted erythrocytes were kindly provided by the blood bank of the University of Tübingen. 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 48 h. Where indicated, erythrocytes were exposed to sorafenib (BAY 43-9006; Enzo, Lörrach, Germany) at the indicated concentrations, which were chosen to be in the range of those encountered in patients following Nexavar® treatment. Where indicated, the erythrocytes were further exposed to N-acetyl-L-cysteine (NAC) and tiron (both from Sigma, Freiburg, Germany), to the kinase inhibitors staurosporine and SB203580 (Enzo), or to the pancaspase inhibitor zVAD (Tocris, Bristol, UK) at the indicated concentrations, which have previously been shown to be effective. In Ca^{2+} -free Ringer solution, 1 mM CaCl_2 was substituted by 1 mM glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA).

FACS analysis of annexin-V-binding and forward scatter

After incubation with or without sorafenib under the indicated experimental condition, 50 μ l 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 fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS calibur (BD, Heidelberg, Germany).

Confocal microscopy and immunofluorescence

For the visualisation of eryptotic erythrocytes, 4 μ l of erythrocyte suspension, incubated under the respective experimental conditions, were stained with FITC-conjugated Annexin-V (1:100 dilution; ImmunoTools, Friesoythe, Germany) in 200 μ l Ringer solution containing 5 mM CaCl_2 . Then the erythrocytes were washed twice and finally re-suspended in 50 μ l of Ringer solution containing 5 mM CaCl_2 . 20 μ l were mounted with Prolong Gold antifade reagent (Invitrogen, Darmstadt, Germany) onto a glass slide, covered with a coverslip, and images were subsequently taken on a Zeiss LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss MicroImaging, Oberkochen, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

Measurement of intracellular Ca^{2+}

After incubation with or without sorafenib under the indicated experimental condition, 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 2 μ M 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 μ l Ringer. Then, Ca^{2+} -dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

Estimation of oxidative stress

Oxidative stress was determined utilizing 2',7' dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 50 μ l 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 μ M. 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 μ l Ringer solution, and ROS-dependent fluorescence intensity was measured in the fluorescence channel FL-1 of a FACS calibur (BD).

Determination of intracellular ATP concentration

For the determination of intracellular erythrocyte ATP, 90 μ l of erythrocyte pellets were incubated for 48 h at 37°C in Ringer solution with or without sorafenib and in Ringer solution with or without extracellular calcium (final hematocrit 5%). Additionally, erythrocytes were also incubated in glucose depleted Ringer solution as a positive control. 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 μ l) 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 the 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.

Determination of ceramide formation

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation with and without sorafenib, cells were stained for 1 h 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 1:5. 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 analysed by flow cytometric analysis in FL-1.

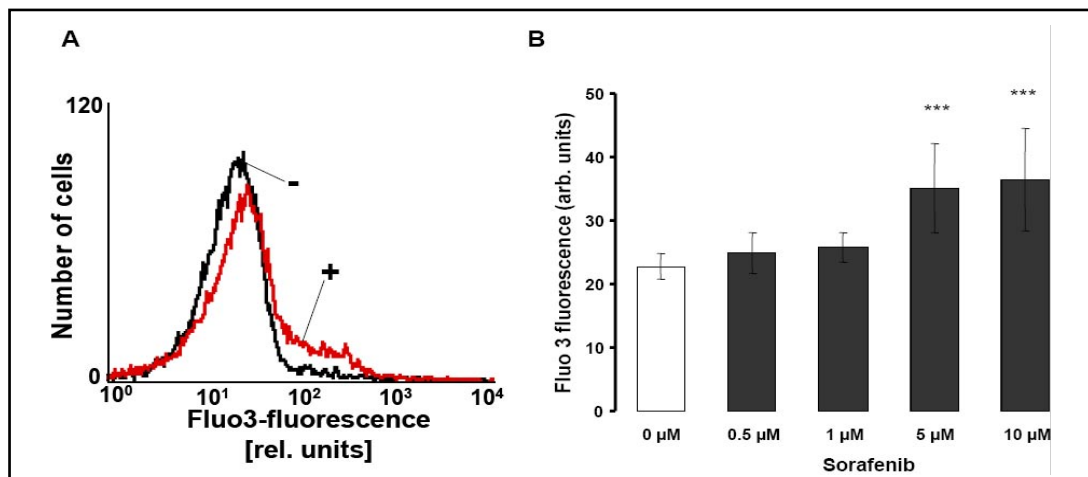


Fig. 1. Effect of sorafenib on Fluo3 fluorescence. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) 10 μ M sorafenib. B. Arithmetic means \pm SD ($n = 10$) of the geo means (arbitrary units) of Fluo3 fluorescence in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) 0.5, 1, 5, or 10 μ M sorafenib. *** ($p < 0.001$) indicates significant difference from the absence of sorafenib (ANOVA).

Measurement of hemolysis

For the determination of hemolysis the samples were centrifuged (3 min at 400 g, room temperature) after incubation, 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.

Statistics

Data are expressed as arithmetic means \pm SD. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and non-parametric Mann-Whitney test. n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to eryptotic effects, the same erythrocyte specimens were used for control and experimental conditions.

Results

Fluo3 fluorescence was employed to determine cytosolic Ca^{2+} activity in FACS analysis. As shown in Fig. 1, the exposure of human erythrocytes to sorafenib was followed by an increase in Fluo3 fluorescence. The effect reached statistical significance at $\geq 5 \mu\text{M}$ sorafenib concentration. An increase in the cytosolic Ca^{2+} concentration is expected to activate Ca^{2+} -sensitive K^+ channels in erythrocytes with subsequent exit of KCl followed by osmotically obliged water and thus cell shrinkage. Cell volume was estimated utilizing forward scatter in FACS analysis. As demonstrated in Fig. 2, sorafenib treatment resulted in a decrease of forward scatter, an effect statistically significant at all concentrations employed ($\geq 0.5 \mu\text{M}$).

Increased cytosolic Ca^{2+} activity further stimulates cell membrane scrambling with phosphatidylserine exposure at the cell surface. To explore, whether sorafenib triggers cell membrane scrambling, phosphatidylserine-exposing erythrocytes were identified using annexin-V-binding. As illustrated in Fig. 3A, the annexin-V-positive erythrocytes were visualized by confocal imaging. Moreover, the number of annexin-V positive erythrocytes was quantified by FACS analysis. As shown in Fig. 3B and 3C, a 48 h exposure to sorafenib increased the percentage of annexin-V binding erythrocytes, an effect reaching statistical significance at $\geq 1 \mu\text{M}$ sorafenib. The sorafenib sensitivity of erythrocyte cell membrane scrambling was seemingly greater than the sorafenib sensitivity of cytosolic Ca^{2+} activity.

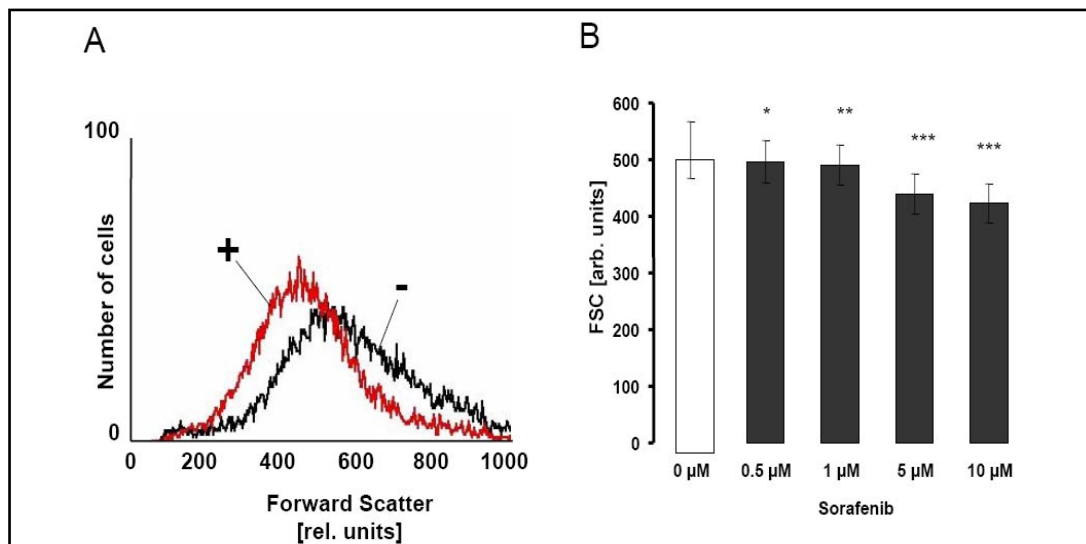


Fig. 2. Effect of sorafenib on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) 10 μ M sorafenib. B. Arithmetic means \pm SD ($n = 10$) of the erythrocyte forward scatter (FSC) following incubation for 48 h in Ringer solution without (white bar) or with (black bars) 0.5, 1, 5, or 10 μ M sorafenib. *, **, *** ($p < 0.05$, $p < 0.01$, $p < 0.001$) indicate significant difference from the absence of sorafenib (ANOVA).

The discrepancy may, however, reflect differences in the sensitivities of annexin-V and Fluo3 fluorescence measurements. Alternatively, sorafenib exerts stronger effects on cell membrane scrambling than on cytosolic Ca^{2+} activity.

Further experiments explored whether sorafenib triggered hemolysis, which was estimated from hemoglobin release into the supernatant. Exposure of erythrocytes for 48 h to sorafenib increased the supernatant hemoglobin concentration, an effect reaching statistical significance at ≥ 5 μ M sorafenib (Fig. 3C).

Additional experiments were performed to explore, whether Ca^{2+} entry accounted for the triggering of erythrocyte cell membrane scrambling. Erythrocytes were exposed to 10 μ M sorafenib in the presence or in the nominal absence of extracellular Ca^{2+} . In the presence of extracellular Ca^{2+} a 48 hours exposure to sorafenib (10 μ M) increased the percentage of annexin-V-binding cells from $2.5 \pm 0.7\%$ ($n = 4$) to $35.9 \pm 6.6\%$ ($n = 4$). In the absence of extracellular Ca^{2+} a 48 hours exposure to sorafenib (10 μ M) increased the percentage annexin-V binding cells from $3.0 \pm 1.0\%$ ($n = 4$) to $37.0 \pm 6.7\%$ ($n = 4$). The increase in annexin-V-binding was not significantly different between the absence and presence of extracellular Ca^{2+} , indicating that Ca^{2+} entry does not account for the cell membrane scrambling of sorafenib-treated erythrocytes.

While those experiments do not rule out that the increase of cytosolic Ca^{2+} activity contributes to the triggering of cell membrane scrambling following sorafenib treatment, they strongly suggest the involvement of cell membrane scrambling mechanisms, which do not require Ca^{2+} entry. Thus, a further series of experiments studied the effect of sorafenib treatment on other cell parameters known to stimulate eryptosis. As a first step, ceramide formation was determined at different time points (0, 6, 12, 24 and 48 h) utilizing anti-ceramide antibodies. As a result, the ceramide-dependent fluorescence intensity was 17.9 ± 1.4 , 21.0 ± 3.3 , 24.1 ± 2.3 , 21.8 ± 0.7 and 17.8 ± 1.7 a.u. ($n = 4$) in the absence of sorafenib; and 19.9 ± 3.6 , 19.8 ± 0.7 , 24.0 ± 1.6 , 22.7 ± 3.9 and 16.2 ± 1.5 a.u. ($n = 4$) following exposure to sorafenib (10 μ M) at the respective time points. The values in the absence and presence of sorafenib were not significantly different. Accordingly, sorafenib did not appreciably stimulate ceramide formation.

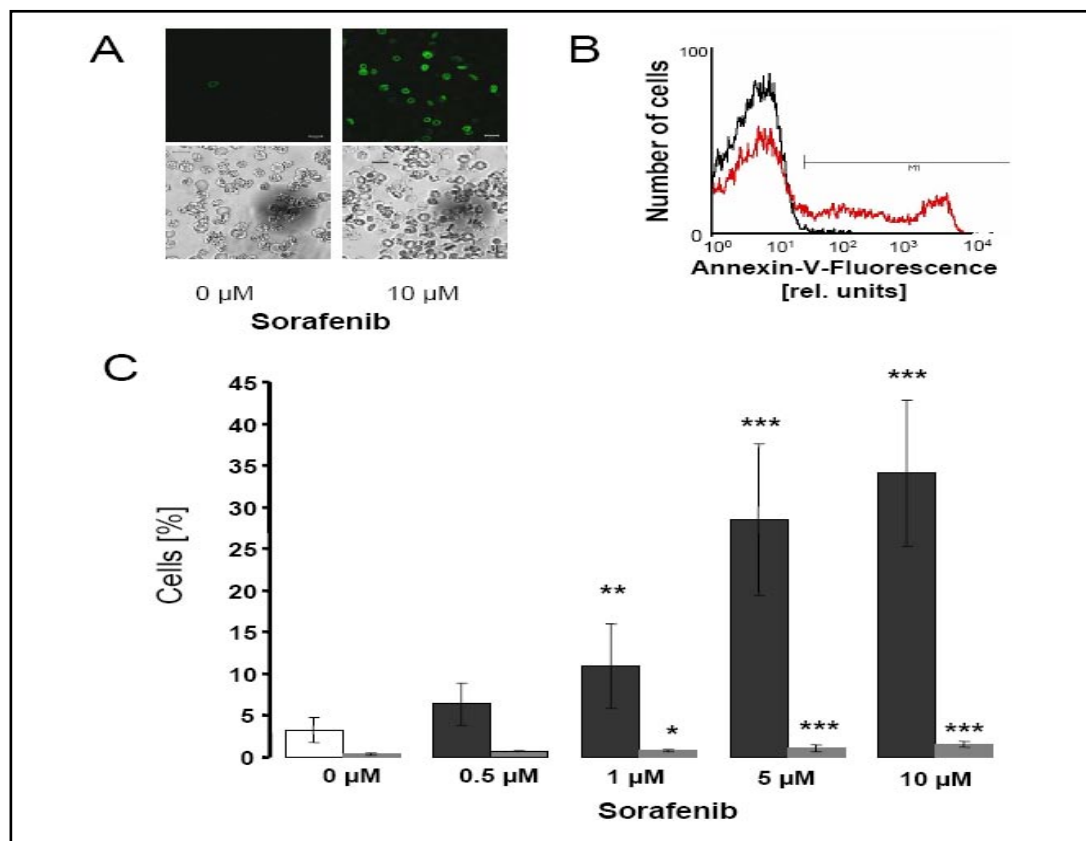


Fig. 3. Effect of sorafenib on phosphatidylserine exposure. A. Confocal microscopy of FITC-dependent fluorescence (upper panels) and transmission light microscopy (lower panels) of human erythrocytes stained with FITC-conjugated Annexin-V following a 48 h incubation in Ringer solution without (left panels) and with (right panels) 10 μM sorafenib. B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (black line) and with (red line) 10 μM sorafenib. C. Arithmetic means ± SD (n = 10) of the percentage of annexin-V-binding erythrocytes following incubation for 48 h in Ringer solution without (white bar) or with (black bars) 0.5, 1, 5, or 10 μM sorafenib. For comparison, arithmetic means ± SD (n = 4) of the percentage of hemolysis is shown as grey bars. *, **, *** (p < 0.05, p < 0.01, p < 0.001) indicates significant difference from the absence of sorafenib (ANOVA).

A next series of experiments explored, whether sorafenib influences cytosolic ATP concentration and thus the energy status of the cell. As a result, following a 48 h exposure to sorafenib (10 μM) cytosolic ATP concentration approached 2.9 ± 0.4 mM while the concentration in the absence of sorafenib was 2.2 ± 0.2 mM, values not significantly different (n = 4). In contrast, a 48 h glucose depletion from the Ringer solution used as a positive control significantly decreased the erythrocyte ATP concentration. Thus, unlike glucose removal, sorafenib did not lead to energy depletion. To confirm these findings, a further series of experiments explored, whether or not sorafenib treatment for shorter time durations (0, 6, 12, and 24 h) may influence cytosolic ATP concentration. As a result, following a 0 h, 6 h, 12 h and 24 h incubation the erythrocyte ATP content was 1.7 ± 0.4 , 1.6 ± 0.4 , 0.8 ± 0.2 and 0.5 ± 0.4 (n = 4), respectively in the absence of sorafenib as well as 2.0 ± 0.2 , 1.8 ± 0.4 , 1.0 ± 0.4 and 0.4 ± 0.5 (n = 4), respectively in the presence of 10 μM sorafenib. The values in the absence and presence of sorafenib were again not significantly different. These results strongly suggest that sorafenib may trigger eryptosis without significantly modifying the energy status of erythrocytes.

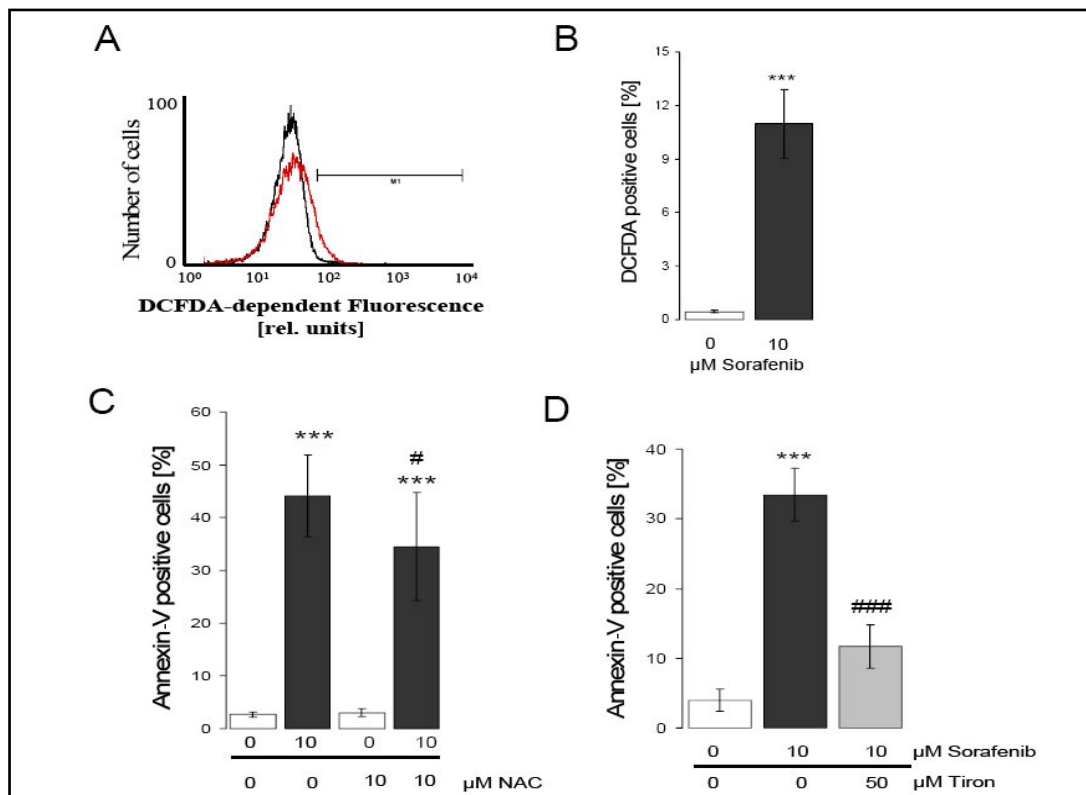
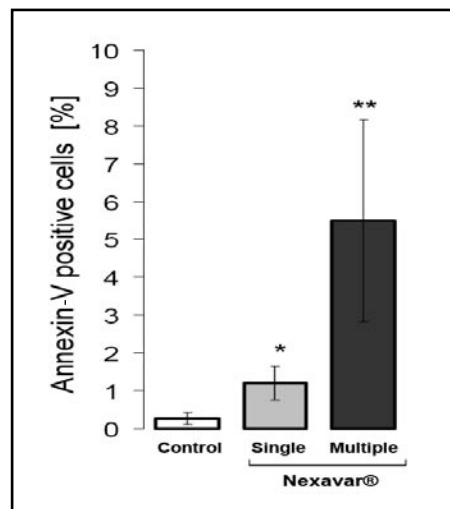


Fig. 4. Effect of sorafenib on the oxidative balance of erythrocytes. A. Original histogram of DCFDA-positive erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) 10 μ M sorafenib. B. Arithmetic means \pm SD ($n = 4$) of the percentage of DCFDA-positive erythrocytes following incubation for 48 h to Ringer solution without (white bar) or with (black bar) 10 μ M sorafenib. *** ($p < 0.001$) indicates significant difference from the absence of sorafenib (ANOVA). C. Arithmetic means \pm SD ($n = 8$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 10 μ M sorafenib in the absence (left bars, 0 NAC) and presence (right bars, 10 NAC) of 10 μ M N-acetyl-cysteine (NAC). *** ($p < 0.001$) indicates significant difference from the absence of sorafenib (ANOVA), # ($p < 0.05$) indicates significant difference from the respective values in the absence of NAC (ANOVA). D. Arithmetic means \pm SD ($n = 4$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bar) 10 μ M sorafenib in the absence (0) and in the presence (grey bar) of 50 μ M tiron. *** ($p < 0.001$) indicates significant difference from the absence of sorafenib (ANOVA), ### ($p < 0.001$) indicates significant difference from treatment with sorafenib alone (ANOVA).

In theory, kinase inhibition by sorafenib could disrupt the inhibition of other kinases and thus lead to activation of those kinases. Thus, additional experiments explored whether the effect of sorafenib could be modified by pharmacological kinase inhibition with staurosporine and SB203580. Both substances have previously been shown to inhibit eryptosis [27, 36]. As a result, a 48 hours exposure to sorafenib (10 μ M) increased the percentage of annexin-V-binding cells from $2.4 \pm 0.4\%$ ($n = 4$) to $31.8 \pm 3.1\%$ ($n = 4$) in the absence and from $4.9 \pm 1.4\%$ ($n = 4$) to $30.3 \pm 3.2\%$ ($n = 4$) in the presence of staurosporine (10 μ M). Moreover, a 48 hours exposure to sorafenib (10 μ M) increased the percentage annexin-V-binding cells from $4.5 \pm 2.1\%$ ($n = 4$) to $28.8 \pm 0.9\%$ ($n = 4$) in the absence and from $3.4 \pm 0.8\%$ ($n = 4$) to $27.0 \pm 1.1\%$ ($n = 4$) in the presence of SB203580 (10 μ M). Thus, neither staurosporine nor SB203580 significantly modified the sorafenib-induced increase in annexin-V-binding.

To explore the potential role of caspases in sorafenib-induced eryptosis, erythrocytes were incubated in the absence and presence of the pancaspase inhibitor zVAD. As a result, a

Fig. 5. *In vivo* eryptosis following treatment with Nexavar®. Arithmetic means \pm SD (n = 6) of the percentage of circulating annexin-V-positive erythrocytes in freshly drawn blood from patients with hepatocellular carcinoma (HCC) prior to treatment (white bar) and treated once (grey bar) or several times (black bar) with Nexavar® (800 mg/day). *, ** (p<0.05, p<0.01) indicates significant difference from untreated patients (non parametric Mann-Whitney test).



48 hours exposure to sorafenib (10 μ M) increased the percentage of annexin-V-binding cells from $2.15 \pm 0.6\%$ (n = 4) to $38.8 \pm 7.5\%$ (n = 4) in the absence and from $4.4 \pm 2.4\%$ (n = 4) to $39.6 \pm 2.6\%$ (n = 4) in the presence of zVAD (10 μ M). Thus, caspase activation appears not to be required for sorafenib-induced eryptosis.

As a next step, the effect of sorafenib treatment on oxidative stress was investigated by measuring 2',7' dichlorodihydrofluorescein diacetate (DCFDA) fluorescence. Exposure of erythrocytes to sorafenib (10 μ M) was followed by a significant increase in DCFDA-dependent fluorescence pointing to oxidative stress (Fig. 4A and 4B). To elucidate the role of oxidative stress in sorafenib-induced eryptosis, the effect of the antioxidant scavenger n-acetyl-cystein (NAC) was tested. As shown in Fig. 4C, NAC significantly blunted the increase in phosphatidylserine-exposing erythrocytes induced by sorafenib. These observations point to oxidative stress in sorafenib-induced suicidal erythrocyte death. Similar to NAC, the antioxidant tiron (50 μ M) blunted the enhanced phosphatidylserine exposure induced by sorafenib (Fig. 4D).

To clarify the *in vivo* significance of sorafenib-induced eryptosis, annexin-V-binding was determined in freshly drawn blood from patients prior to and following single or multiple administrations of Nexavar®. As illustrated in Fig. 5, the sorafenib treatment was indeed followed by a significant increase in the percentage of circulating annexin-V-positive erythrocytes *in vivo*. Thus, sorafenib stimulates eryptosis *in vivo*.

Discussion

The present study discloses a novel effect of sorafenib, i.e. the stimulation of suicidal erythrocyte death or eryptosis. Sorafenib exposure triggers cell membrane scrambling and leads to cell shrinkage. The concentration required for the effect on cell membrane scrambling is well in the range of therapeutic plasma concentrations [37]. Accordingly, the percentage of eryptotic cells increased following Nexavar® treatment. Only a fraction of the treated erythrocytes underwent eryptosis pointing to differences in susceptibility of the erythrocyte population. As shown recently [38], the susceptibility to eryptosis increases with erythrocyte age.

Sorafenib increased Fluo3 fluorescence reflecting an elevated cytosolic Ca^{2+} concentration. Ca^{2+} entry with subsequent increase in the cytosolic Ca^{2+} activity requires the activation of non-selective cation channels, which have previously been shown to involve TRPC6 [21]. Activators of the cation channels include oxidative stress [39].

Sorafenib exposure further resulted in cell shrinkage, as apparent from forward scatter. The cell shrinkage following sorafenib treatment most likely results from activation of Ca^{2+} -

sensitive K⁺ channels [23, 40]. Opening of those channels fosters exit of K⁺ following its chemical gradient, resulting in cell membrane hyperpolarisation, Cl⁻ exit and thus cellular loss of KCl with osmotically obliged water [24].

As shown previously [25, 41, 42], an increase in the cytosolic Ca²⁺ activity stimulates cell membrane scrambling leading to phosphatidylserine exposure at the erythrocyte surface. Surprisingly, though, the nominal absence of extracellular Ca²⁺ did not abrogate the scrambling effect of sorafenib. Ca²⁺ entry is thus not required for sorafenib-induced phosphatidylserine exposure. This observation does not rule out the possibility that the increase of cytosolic Ca²⁺ activity contributes to sorafenib induced eryptosis but clearly demonstrates that additional mechanisms must be involved in sorafenib induced cell membrane scrambling, which are effective even in the absence of Ca²⁺ entry.

Sorafenib did not significantly modify the formation of ceramide, which has previously been shown to stimulate cell membrane scrambling in erythrocytes [26, 43] and nucleated cells [44].

As cell membrane scrambling is, in addition, triggered by energy depletion [27], additional experiments explored, whether or not sorafenib decreased cytosolic ATP concentration. As a result, sorafenib did not significantly modify cytosolic ATP content. The present experiments further demonstrate that cell membrane scrambling following sorafenib exposure does not require activation of caspases or activation of staurosporine or SB203580 sensitive kinases. Again, the observations do not rule out the activation of caspases and those kinases, but point to the involvement of further mechanisms in the triggering of phosphatidylserine exposure.

Sorafenib-induced apoptosis of nucleated cells was previously shown to be modulated by oxidative stress [45, 46]. The present observations reveal that exposure of erythrocytes to sorafenib indeed enhances oxidative stress and that the antioxidant N-acetyl-L-cysteine significantly blunts eryptosis induced by sorafenib. Thus, oxidative stress is required for full activation of cell membrane scrambling following sorafenib treatment.

Similar to sorafenib, a wide variety of xenobiotics trigger eryptosis [28, 47-56]. Moreover, enhanced eryptosis is encountered in several clinical disorders [20], including diabetes [32, 57, 58], renal insufficiency [59], hemolytic uremic syndrome [60], sepsis [61], sickle cell disease [62], malaria [63-67], Wilson's disease [67], iron deficiency [68], phosphate depletion [69], APC gene mutations [70] and presumably metabolic syndrome [71]. Phosphatidylserine-exposing erythrocytes are rapidly cleared from circulating blood. Excessive eryptosis may thus lead to anemia [20]. The eryptosis inducing xenobiotics or disorders may thus be expected to aggravate sorafenib induced anemia.

Phosphatidylserine-exposing erythrocytes bind to the luminal face of endothelial cells thus impairing microcirculation [72-76]. Eryptotic erythrocytes further stimulate blood clotting [72, 77, 78]. Thus, in theory, sorafenib induced eryptosis may predispose to thrombosis.

The present observations provide an explanation for the anemia observed following Nexavar® treatment of patients with hepatocellular carcinoma. The observations do not rule out further causes of anemia, such as bleeding or compromised erythropoiesis. It should be kept in mind that the iron deficiency following blood loss leads to formation of erythrocytes particularly prone to undergo premature eryptosis [68]. To the extent that the anemia is caused largely by eryptosis, the enhanced formation of phosphatidylserine exposing erythrocytes may further jeopardize microcirculation with the risk of thrombosis. Eryptosis may be particularly prevalent in patients suffering from clinical conditions or treated with drugs fostering eryptosis (see above). Those patients may benefit from additional treatment with substances known to inhibit eryptosis [20]. It is intriguing to speculate that particularly the administration of antioxidants may attenuate the eryptosis and anemia during treatment with Nexavar® and thus prove beneficial in those patients with excessive eryptosis. On the other hand, it cannot be ruled out that the full anti-cancer effect of Nexavar® requires oxidative stress.

In conclusion, sorafenib triggers eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling of erythrocytes and may account for the development of clinically relevant anemia in Nexavar® chemotherapy.

Competing interests

The authors declare, in relation to this manuscript, that they have no financial competing interests (political, personal, religious, ideological, academic, intellectual, commercial or any other).

Authors' contributions

AL organized the study and performed the *in vivo* experiments. NS, KJ, EL, and MZ performed the *in vitro* experiments. NS executed the statistical analysis and prepared the figures. CZ performed confocal imaging. AP and MB provided the HCC patient blood samples. AL, MF and SMQ coordinated and supervised the experiments. FL designed the study and drafted the manuscript. All authors carefully read and approved the manuscript.

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References

- 1 Abou-Alfa GK, Saltz LB: Doxorubicin and sorafenib for treatment of advanced hepatocellular cancer. *Gastroenterology* 2011;141:e19-e20.
- 2 Duntas LH, Bernardini R: Sorafenib: rays of hope in thyroid cancer. *Thyroid* 2010;20:1351-1358.
- 3 Escudier B: Sorafenib for the management of advanced renal cell carcinoma. *Expert Rev Anticancer Ther* 2011;11:825-836.
- 4 Gounder MM, Lefkowitz RA, Keohan ML, D'Adamo DR, Hameed M, Antonescu CR, Singer S, Stout K, Ahn L, Maki RG: Activity of Sorafenib against desmoid tumor/deep fibromatosis. *Clin Cancer Res* 2011;17:4082-4090.
- 5 Guan YS, He Q: Sorafenib: activity and clinical application in patients with hepatocellular carcinoma. *Expert Opin Pharmacother* 2011;12:303-313.
- 6 Semrad TJ, Gandara DR, Lara PN Jr: Enhancing the clinical activity of sorafenib through dose escalation: rationale and current experience. *Ther Adv Med Oncol* 2011;3:95-100.
- 7 Zhang Z, Zhou X, Shen H, Wang D, Wang Y: Phosphorylated ERK is a potential predictor of sensitivity to sorafenib when treating hepatocellular carcinoma: evidence from an *in vitro* study. *BMC Med* 2009;7:41.
- 8 Zhu AX: Predicting the response to sorafenib in hepatocellular carcinoma: where is the evidence for phosphorylated extracellular signaling-regulated kinase (pERK)? *BMC Med* 2009;7:42.
- 9 Zhang J, Gold KA, Kim E: Sorafenib in non-small cell lung cancer. *Expert Opin Investig Drugs* 2012; in press.
- 10 Matsuda Y, Fukumoto M: Sorafenib: complexities of Raf-dependent and Raf-independent signaling are now unveiled. *Med Mol Morphol* 2011;44:183-189.
- 11 Chapuy B, Schuelper N, Panse M, Dohm A, Hand E, Schroers R, Truemper L, Wulf GG: Multikinase inhibitor sorafenib exerts cytotoxic efficacy against Non-Hodgkin lymphomas associated with inhibition of MAPK14 and AKT phosphorylation. *Br J Haematol* 2011;152:401-412.

- 12 Fecteau JF, Bharati IS, O'hayre M, Handel TM, Kipps TJ, Messmer D: Sorafenib-induced apoptosis of chronic lymphocytic leukemia cells is associated with downregulation of RAF and Mcl-1. *Mol Med* 2012;18:19-28.
- 13 Huynh H, Choo SP, Toh HC, Tai WM, Chung AY, Chow PK, Ong R, Soo KC: Comparing the Efficacy of Sunitinib with Sorafenib in Xenograft Models of Human Hepatocellular Carcinoma: Mechanistic Explanation. *Curr Cancer Drug Targets* 2011;11:944-953.
- 14 Shi YH, Ding ZB, Zhou J, Hui B, Shi GM, Ke AW, Wang XY, Dai Z, Peng YF, Gu CY, Qiu SJ, Fan J: Targeting autophagy enhances sorafenib lethality for hepatocellular carcinoma via ER stress-related apoptosis. *Autophagy* 2011;7:1159-1172.
- 15 Fernando J, Sancho P, Fernandez-Rodriguez CM, Lledo JL, Caja L, Campbell J, Fausto N, Fabregat I: Sorafenib sensitizes hepatocellular carcinoma cells to physiological apoptotic stimuli. *J Cell Physiol* 2012;227:1319-1325.
- 16 Huber S, Oelsner M, Decker T, zum Buschenfelde CM, Wagner M, Lutzny G, Kuhnt T, Schmidt B, Oostendorp RA, Peschel C, Ringshausen I: Sorafenib induces cell death in chronic lymphocytic leukemia by translational downregulation of Mcl-1. *Leukemia* 2011;25:838-847.
- 17 Hartmann JT, Haap M, Kopp HG, Lipp HP: Tyrosine kinase inhibitors - a review on pharmacology, metabolism and side effects. *Curr Drug Metab* 2009;10:470-481.
- 18 Mancuso A, Airoidi A, Vigano R, Pinzello G: Fatal gastric bleeding during sorafenib treatment for hepatocellular carcinoma recurrence after liver transplantation. *Dig Liver Dis* 2011;43:754.
- 19 Rombola F, Caravetta A, Mollo F, Spinoso A, Peluso L, Guarino R: Sorafenib, risk of bleeding and spontaneous rupture of hepatocellular carcinoma. A clinical case. *Acta Medica (Hradec Kralove)* 2011;54:177-179.
- 20 Lang F, Gulbins E, Lerche H, Huber SM, Kempe DS, Foller M: Eryptosis, a window to systemic disease. *Cell Physiol Biochem* 2008;22:373-380.
- 21 Foller M, Kasinathan RS, Koka S, Lang C, Shumilina E, Birnbaumer L, Lang F, Huber SM: TRPC6 contributes to the Ca²⁺ leak of human erythrocytes. *Cell Physiol Biochem* 2008;21:183-192.
- 22 Foller M, Sopjani M, Koka S, Gu S, Mahmud H, Wang K, Floride E, Schleicher E, Schulz E, Munzel T, Lang F: Regulation of erythrocyte survival by AMP-activated protein kinase. *FASEB J* 2009;23:1072-1080.
- 23 Brugnara C, de Franceschi L, Alper SL: Inhibition of Ca²⁺-dependent K⁺ transport and cell dehydration in sickle erythrocytes by clotrimazole and other imidazole derivatives. *J Clin Invest* 1993;92:520-526.
- 24 Lang PA, Kaiser S, Myssina S, Wieder T, Lang F, Huber SM: Role of Ca²⁺-activated K⁺ channels in human erythrocyte apoptosis. *Am J Physiol Cell Physiol* 2003;285:C1553-C1560.
- 25 Berg CP, Engels IH, Rothbart A, Lauber K, Renz A, Schlosser SF, Schulze-Osthoff K, Wesselborg S: Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. *Cell Death Differ* 2001;8:1197-1206.
- 26 Lang F, Gulbins E, Lang PA, Zappulla D, Foller M: Ceramide in suicidal death of erythrocytes. *Cell Physiol Biochem* 2010;26:21-28.
- 27 Klarl BA, Lang PA, Kempe DS, Niemoeller OM, Akel A, Sobiesiak M, Eisele K, Podolski M, Huber SM, Wieder T, Lang F: Protein kinase C mediates erythrocyte "programmed cell death" following glucose depletion. *Am J Physiol Cell Physiol* 2006;290:C244-C253.
- 28 Bhavsar SK, Bobbala D, Xuan NT, Foller M, Lang F: Stimulation of suicidal erythrocyte death by alpha-lipoic acid. *Cell Physiol Biochem* 2010;26:859-868.
- 29 Foller M, Huber SM, Lang F: Erythrocyte programmed cell death. *IUBMB Life* 2008;60:661-668.
- 30 Foller M, Mahmud H, Gu S, Wang K, Floride E, Kucherenko Y, Luik S, Laufer S, Lang F: Participation of leukotriene C(4) in the regulation of suicidal erythrocyte death. *J Physiol Pharmacol* 2009;60:135-143.
- 31 Lau IP, Chen H, Wang J, Ong HC, Leung KC, Ho HP, Kong SK: In vitro effect of CTAB- and PEG-coated gold nanorods on the induction of eryptosis/erythroptosis in human erythrocytes. *Nanotoxicology* 2011, DOI: [10.3109/17435390.2011.625132](https://doi.org/10.3109/17435390.2011.625132).
- 32 Maellaro E, Leoncini S, Moretti D, Del Bello B, Tanganelli I, De Felice C, Ciccoli L: Erythrocyte caspase-3 activation and oxidative imbalance in erythrocytes and in plasma of type 2 diabetic patients. *Acta Diabetol* 2011, DOI: [10.1007/s00592-011-0274-0](https://doi.org/10.1007/s00592-011-0274-0).
- 33 Foller M, Feil S, Ghoreschi K, Koka S, Gerling A, Thunemann M, Hofmann F, Schuler B, Vogel J, Pichler B, Kasinathan RS, Nicolay JP, Huber SM, Lang F, Feil R: Anemia and splenomegaly in cGKI-deficient mice. *Proc Natl Acad Sci U S A* 2008;105:6771-6776.
- 34 Bhavsar SK, Gu S, Bobbala D, Lang F: Janus kinase 3 is expressed in erythrocytes, phosphorylated upon energy depletion and involved in the regulation of suicidal erythrocyte death. *Cell Physiol Biochem* 2011;27:547-556.

- 35 Zelenak C, Eberhard M, Jilani K, Qadri SM, Macek B, Lang F: Protein Kinase CK1 α Regulates Erythrocyte Survival. *Cell Physiol Biochem* 2012;29:171-180.
- 36 Gatidis S, Zelenak C, Fajol A, Lang E, Jilani K, Michael D, Qadri SM, Lang F: p38 MAPK activation and function following osmotic shock of erythrocytes. *Cell Physiol Biochem* 2011;28:1279-1286.
- 37 Bobin-Dubigeon C, Heurgue-Berlot A, Bouche O, Amiand MB, Le Guellec C, Bard JM: A New Rapid and Sensitive LC-MS Assay for the Determination of Sorafenib in Plasma: Application to a Patient Undergoing Hemodialysis. *Ther Drug Monit* 2011;33:705-710.
- 38 Ghashghaieina M, Cluitmans JC, Akel A, Dreischer P, Toulany M, Koberle M, Skabytska Y, Saki M, Biedermann T, Duszenko M, Lang F, Wieder T, Bosman GJ: The impact of erythrocyte age on eryptosis. *Br J Haematol* 2012;157:606-614.
- 39 Brand VB, Sandu CD, Duranton C, Tanneur V, Lang KS, Huber SM, Lang F: Dependence of Plasmodium falciparum in vitro growth on the cation permeability of the human host erythrocyte. *Cell Physiol Biochem* 2003;13:347-356.
- 40 Bookchin RM, Ortiz OE, Lew VL: Activation of calcium-dependent potassium channels in deoxygenated sickled red cells. *Prog Clin Biol Res* 1987;240:193-200.
- 41 Bratosin D, Estaquier J, Petit F, Arnoult D, Quatannens B, Tissier JP, Slomianny C, Sartiaux C, Alonso C, Huart JJ, Montreuil J, Ameisen JC: Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. *Cell Death Differ* 2001;8:1143-1156.
- 42 Lang KS, Duranton C, Poehlmann H, Myssina S, Bauer C, Lang F, Wieder T, Huber SM: Cation channels trigger apoptotic death of erythrocytes. *Cell Death Differ* 2003;10:249-256.
- 43 Lang KS, Myssina S, Brand V, Sandu C, Lang PA, Berchtold S, Huber SM, Lang F, Wieder T: Involvement of ceramide in hyperosmotic shock-induced death of erythrocytes. *Cell Death Differ* 2004;11:231-243.
- 44 Kornhuber J, Tripal P, Reichel M, Muhle C, Rhein C, Muehlbacher M, Groemer TW, Gulbins E: Functional Inhibitors of Acid Sphingomyelinase (FIASMAS): a novel pharmacological group of drugs with broad clinical applications. *Cell Physiol Biochem* 2010;26:9-20.
- 45 Caraglia M, Giuberti G, Marra M, Addeo R, Montella L, Murolo M, Sperlongano P, Vincenzi B, Naviglio S, Prete SD, Abbruzzese A, Stiuso P: Oxidative stress and ERK1/2 phosphorylation as predictors of outcome in hepatocellular carcinoma patients treated with sorafenib plus octreotide LAR. *Cell Death Dis* 2011;2:e150.
- 46 Chiou JF, Tai CJ, Wang YH, Liu TZ, Jen YM, Shiao CY: Sorafenib induces preferential apoptotic killing of a drug- and radio-resistant Hep G2 cells through a mitochondria-dependent oxidative stress mechanism. *Cancer Biol Ther* 2009;8:1904-1913.
- 47 Bhavsar SK, Eberhard M, Bobbala D, Lang F: Monensin induced suicidal erythrocyte death. *Cell Physiol Biochem* 2010;25:745-752.
- 48 Braun M, Foller M, Gulbins E, Lang F: Eryptosis triggered by bismuth. *Biometals* 2009;22:453-460.
- 49 Eberhard M, Ferlinz K, Alizzi K, Cacciato PM, Faggio C, Foller M, Lang F: FTY720-induced suicidal erythrocyte death. *Cell Physiol Biochem* 2010;26:761-766.
- 50 Ghashghaieina M, Toulany M, Saki M, Bobbala D, Fehrenbacher B, Rupec R, Rodemann HP, Ghoreschi K, Rocken M, Schaller M, Lang F, Wieder T: The NF κ B Pathway Inhibitors Bay 11-7082 and Parthenolide Induce Programmed Cell Death in Anucleated Erythrocytes. *Cell Physiol Biochem* 2011;27:45-54.
- 51 Lang E, Jilani K, Zelenak C, Pasham V, Bobbala D, Qadri SM, Lang F: Stimulation of suicidal erythrocyte death by benzethonium. *Cell Physiol Biochem* 2011;28:347-354.
- 52 Mahmud H, Foller M, Lang F: Arsenic-induced suicidal erythrocyte death. *Arch Toxicol* 2009;83:107-113.
- 53 Mahmud H, Mauro D, Qadri SM, Foller M, Lang F: Triggering of suicidal erythrocyte death by amphotericin B. *Cell Physiol Biochem* 2009;24:263-270.
- 54 Mahmud H, Mauro D, Foller M, Lang F: Inhibitory effect of thymol on suicidal erythrocyte death. *Cell Physiol Biochem* 2009;24:407-414.
- 55 Qadri SM, Bauer J, Zelenak C, Mahmud H, Kucherenko Y, Lee SH, Ferlinz K, Lang F: Sphingosine but not sphingosine-1-phosphate stimulates suicidal erythrocyte death. *Cell Physiol Biochem* 2011;28:339-346.
- 56 Qadri SM, Kucherenko Y, Zelenak C, Jilani K, Lang E, Lang F: Dicoumarol activates Ca²⁺-permeable cation channels triggering erythrocyte cell membrane scrambling. *Cell Physiol Biochem* 2011;28:857-864.
- 57 Calderon-Salinas JV, Munoz-Reyes EG, Guerrero-Romero JF, Rodriguez-Moran M, Bracho-Riquelme RL, Carrera-Gracia MA, Quintanar-Escorza MA: Eryptosis and oxidative damage in type 2 diabetic mellitus patients with chronic kidney disease. *Mol Cell Biochem* 2011;357:171-179.

- 58 Nicolay JP, Schneider J, Niemoeller OM, Artunc F, Portero-Otin M, Haik G Jr, Thornalley PJ, Schleicher E, Wieder T, Lang F: Stimulation of suicidal erythrocyte death by methylglyoxal. *Cell Physiol Biochem* 2006;18:223-232.
- 59 Myssina S, Huber SM, Birka C, Lang PA, Lang KS, Friedrich B, Risler T, Wieder T, Lang F: Inhibition of erythrocyte cation channels by erythropoietin. *J Am Soc Nephrol* 2003;14:2750-2757.
- 60 Lang PA, Beringer O, Nicolay JP, Amon O, Kempe DS, Hermle T, Attanasio P, Akel A, Schafer R, Friedrich B, Risler T, Baur M, Olbricht CJ, Zimmerhackl LB, Zipfel PF, Wieder T, Lang F: Suicidal death of erythrocytes in recurrent hemolytic uremic syndrome. *J Mol Med* 2006;84:378-388.
- 61 Kempe DS, Akel A, Lang PA, Hermle T, Biswas R, Muresanu J, Friedrich B, Dreischer P, Wolz C, Schumacher U, Peschel A, Gotz F, Doring G, Wieder T, Gulbins E, Lang F: Suicidal erythrocyte death in sepsis. *J Mol Med* 2007;85:273-281.
- 62 Lang PA, Kasinathan RS, Brand VB, Duranton C, Lang C, Koka S, Shumilina E, Kempe DS, Tanneur V, Akel A, Lang KS, Foller M, Kun JF, Kreamsner PG, Wesselborg S, Laufer S, Clemen CS, Herr C, Noegel AA, Wieder T, Gulbins E, Lang F, Huber SM: Accelerated clearance of Plasmodium-infected erythrocytes in sickle cell trait and annexin-A7 deficiency. *Cell Physiol Biochem* 2009;24:415-428.
- 63 Siraskar B, Ballal A, Bobbala D, Foller M, Lang F: Effect of amphotericin B on parasitemia and survival of plasmodium berghei-infected mice. *Cell Physiol Biochem* 2010;26:347-354.
- 64 Bobbala D, Alesutan I, Foller M, Huber SM, Lang F: Effect of anandamide in Plasmodium Berghei-infected mice. *Cell Physiol Biochem* 2010;26:355-362.
- 65 Foller M, Bobbala D, Koka S, Huber SM, Gulbins E, Lang F: Suicide for survival--death of infected erythrocytes as a host mechanism to survive malaria. *Cell Physiol Biochem* 2009;24:133-140.
- 66 Koka S, Bobbala D, Lang C, Boini KM, Huber SM, Lang F: Influence of paclitaxel on parasitemia and survival of Plasmodium berghei infected mice. *Cell Physiol Biochem* 2009;23:191-198.
- 67 Lang PA, Schenck M, Nicolay JP, Becker JU, Kempe DS, Lupescu A, Koka S, Eisele K, Klarl BA, Rubben H, Schmid KW, Mann K, Hildenbrand S, Heftner H, Huber SM, Wieder T, Erhardt A, Haussinger D, Gulbins E, Lang F: Liver cell death and anemia in Wilson disease involve acid sphingomyelinase and ceramide. *Nat Med* 2007;13:164-170.
- 68 Kempe DS, Lang PA, Duranton C, Akel A, Lang KS, Huber SM, Wieder T, Lang F: Enhanced programmed cell death of iron-deficient erythrocytes. *FASEB J* 2006;20:368-370.
- 69 Birka C, Lang PA, Kempe DS, Hoefling L, Tanneur V, Duranton C, Nammi S, Henke G, Myssina S, Krikov M, Huber SM, Wieder T, Lang F: Enhanced susceptibility to erythrocyte "apoptosis" following phosphate depletion. *Pflugers Arch* 2004;448:471-477.
- 70 Qadri SM, Mahmud H, Lang E, Gu S, Bobbala D, Zelenak C, Jilani K, Siegfried A, Foller M, Lang F: Enhanced suicidal erythrocyte death in mice carrying a loss of function mutation of the Adenomatous Polyposis Coli gene. *J Cell Mol Med* 2012;16:1085-1093.
- 71 Zappulla D: Environmental stress, erythrocyte dysfunctions, inflammation, and the metabolic syndrome: adaptations to CO₂ increases? *J Cardiometab Syndr* 2008;3:30-34.
- 72 Andrews DA, Low PS: Role of red blood cells in thrombosis. *Curr Opin Hematol* 1999;6:76-82.
- 73 Closse C, Dachary-Prigent J, Boisseau MR: Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. *Br J Haematol* 1999;107:300-302.
- 74 Gallagher PG, Chang SH, Rettig MP, Neely JE, Hillery CA, Smith BD, Low PS: Altered erythrocyte endothelial adherence and membrane phospholipid asymmetry in hereditary hydrocytosis. *Blood* 2003;101:4625-4627.
- 75 Pandolfi A, Di Pietro N, Siroli V, Giardinelli A, Di Silvestre S, Amoroso L, Di Tomo P, Capani F, Consoli A, Bonomini M: Mechanisms of uremic erythrocyte-induced adhesion of human monocytes to cultured endothelial cells. *J Cell Physiol* 2007;213:699-709.
- 76 Wood BL, Gibson DE, Tait JF: Increased erythrocyte phosphatidylserine exposure in sickle cell disease: flow-cytometric measurement and clinical associations. *Blood* 1996;88:1873-1880.
- 77 Chung SM, Bae ON, Lim KM, Noh JY, Lee MY, Jung YS, Chung JH: Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant microvesicle generation in human erythrocytes. *Arterioscler Thromb Vasc Biol* 2007;27:414-421.
- 78 Zwaal RF, Comfurius P, Bevers EM: Surface exposure of phosphatidylserine in pathological cells. *Cell Mol Life Sci* 2005;62:971-988.