Enhanced Erythrocyte Membrane Exposure of Phosphatidylserine Following Sorafenib Treatment: An \textit{in vivo} and \textit{in vitro} Study

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Key Words
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Abstract
\textbf{Background:} Sorafenib (Nexavar\textsuperscript{®}), a polytyrosine kinase inhibitor, stimulates apoptosis and is thus widely used for chemotherapy in hepatocellular carcinoma (HCC). Hematological side effects of Nexavar\textsuperscript{®} 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\textsuperscript{2+}-activity ([Ca\textsuperscript{2+}]\textsubscript{i}), formation of ceramide, ATP-depletion and oxidative stress. The present study explored, whether sorafenib triggers eryptosis \textit{in vitro} and \textit{in vivo}. \textbf{Methods:} [Ca\textsuperscript{2+}], 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. \textbf{Results:} A 48 h exposure of erythrocytes to sorafenib (≥0.5µM) significantly increased Fluo 3 fluorescence, decreased forward scatter, increased annexin-V-binding and triggered slight hemolysis (≥5µM), 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\textsuperscript{®} chemotherapy in HCC patients significantly enhanced the number of phosphatidylserine-exposing erythrocytes. \textbf{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\textsuperscript{®}-based chemotherapy.
Introduction

Sorafenib (Nexavar®) is a polytyrosine kinase 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 Ca²⁺ entry through Ca²⁺-permeable cation channels [21, 22]. An increase in the cytosolic Ca²⁺ concentration is followed by activation of Ca²⁺-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 Ca²⁺ 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 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl₂; 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 Ca²⁺-free Ringer solution, 1 mM CaCl₂ 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 µl cell suspension was washed in Ringer solution containing 5 mM CaCl\textsubscript{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 µl of erythrocyte suspension, incubated under the respective experimental conditions, were stained with FITC-conjugated Annexin-V (1:100 dilution; ImmunoTools, Friesoythe, Germany) in 200 µl Ringer solution containing 5 mM CaCl\textsubscript{2}. Then the erythrocytes were washed twice and finally re-suspended in 50 µl of Ringer solution containing 5 mM CaCl\textsubscript{2}. 20 µl 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 MicrolImaging, Oberkochen, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

**Measurement of intracellular Ca\textsuperscript{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\textsubscript{2} and 2 µM Fluo-3/AM. The cells were incubated at 37°C for 30 min and washed twice in Ringer solution containing 5 mM CaCl\textsubscript{2}. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer. Then, Ca\textsuperscript{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 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 in the fluorescence channel FL-1 of a FACS calibur (BD).

**Determination of intracellular ATP concentration**

For the determination of intracellular erythrocyte ATP, 90 µl 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\textsubscript{4} (5%). After centrifugation, an aliquot of the supernatant (400 µl) was adjusted to pH 7.7 by addition of saturated KHCO\textsubscript{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 µg/ml anti-ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:5. 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 (Pharminimum, 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.
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 ± 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 ≥5 µM 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 (≥0.5 µM).

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 ≥1 µM sorafenib. The sorafenib sensitivity of erythrocyte cell membrane scrambling was seemingly greater than the sorafenib sensitivity of cytosolic Ca\(^{2+}\) activity.
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 µM 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 µM 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 µM) increased the percentage of annexin-V-binding cells from 2.5 ± 0.7% (n = 4) to 35.9 ± 6.6% (n = 4). In the absence of extracellular Ca\(^{2+}\) a 48 hours exposure to sorafenib (10 µM) increased the percentage annexin-V binding cells from 3.0 ± 1.0% (n = 4) to 37.0 ± 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 epyrosis. 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, 21.4 ± 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 µM) 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.
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.
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 µM) increased the percentage of annexin-V-binding erythrocytes from 2.4 ± 0.4% (n = 4) to 31.8 ± 3.1% (n = 4) in the absence and from 4.9 ± 1.4% (n = 4) to 30.3 ± 3.2% (n = 4) in the presence of staurosporine (10 µM). Moreover, a 48 hours exposure to sorafenib (10 µM) increased the percentage annexin-V-binding erythrocytes from 4.5 ± 2.1% (n = 4) to 28.8 ± 0.9% (n = 4) in the absence and from 3.4 ± 0.8% (n = 4) to 27.0 ± 1.1% (n = 4) in the presence of SB203580 (10 µM). 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
48 hours exposure to sorafenib (10 µM) increased the percentage of annexin-V-binding cells from 2.15 ± 0.6% (n = 4) to 38.8 ± 7.5% (n = 4) in the absence and from 4.4 ± 2.4% (n = 4) to 39.6 ± 2.6% (n = 4) in the presence of zVAD (10 µM). 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 µM) 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 µM) 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 apoptotic 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 Ca²⁺ concentration. Ca²⁺ entry with subsequent increase in the cytosolic Ca²⁺ 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 Ca²⁺-
sensitive K⁺ channels [23, 40]. Opening of those channels fosters exit of K⁺ following its chemical gradient, resulting in cell membrane hyperpolarization, 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 caspasases or activation of staurosporine or SB203580 sensitive kinases. Again, the observations do not rule out the activation 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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