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Original Paper

Triggering of Suicidal Erythrocyte Death by Regorafenib

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Key Words
Phosphatidylserine • Cell volume • Eryptosis • Ionomycin • Calcium

Abstract
Background/Aims: The multikinase inhibitor regorafenib is utilized for the treatment of malignancy. The substance is effective in part by triggering suicidal death or apoptosis of tumor cells. Side effects of regorafenib include anemia. At least in theory, regorafenib induced anemia could result from stimulated suicidal erythrocyte death or eryptosis, characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Triggers of eryptosis include increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), oxidative stress and ceramide. The present study explored, whether regorafenib induces eryptosis and, if so, whether it is effective up- and/or downstream of Ca^{2+}. ***Methods:*** To this end, phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, $[Ca^{2+}]_i$ from Fluo3-fluorescence, ROS formation from DCFDA dependent fluorescence, and ceramide abundance utilizing specific antibodies. ***Results:*** A 48 hours exposure of human erythrocytes to regorafenib (≥ 0.5 µg/ml) significantly increased the percentage of annexin-V-binding cells, significantly decreased forward scatter (≥ 1.25 µg/ml), but did not significantly increase Fluo3-fluorescence, DCFDA fluorescence or ceramide abundance. The effect of regorafenib on annexin-V-binding and forward scatter was not significantly blunted by removal of extracellular Ca^{2+}. Regorafenib (5 µg/ml) significantly augmented the increase of annexin-V-binding, but significantly blunted the decrease of forward scatter following treatment with the Ca^{2+} ionophore ionomycin. ***Conclusions:*** Regorafenib triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect at least in part downstream of Ca^{2+}.

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Introduction

The multitargeted tyrosine kinase inhibitor regorafenib with antiangiogenic activity [1-8] is used for the treatment of colorectal cancer and gastrointestinal stromal tumors [1-23] as well as other malignancies including hepatocellular carcinoma [3, 24], renal cell carcinoma [3], and soft tissue sarcoma [3]. Kinases inhibited by regorafenib include VEGFR1-3, c-KIT, TIE-2, PDGFR-β, FGFR-1, RET, RAF-1, BRAF and p38 MAP kinase [8]. Regorafenib is partially effective by inducing apoptosis [6, 25-35] or necrosis [36] of tumor cells. Observed side effects of regorafenib include anemia [37, 38].

In theory, anemia could result from eryptosis [39], the suicidal death of erythrocytes characterized by cell shrinkage [40] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [39]. Triggers of eryptosis include increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) [39], ceramide [41], oxidative stress [39], energy depletion [39], activated caspases [39, 42, 43], stimulated activity of casein kinase 1α, Janus-activated kinase JAK3, protein kinase C, p38 kinase and PAK2 kinase [39], as well as impaired activity of AMP activated kinase AMPK, cGMP-dependent protein kinase, and sorafenib/sunitinib sensitive kinases [39]. Eryptosis is triggered by multiple xenobiotics [39, 44-74].

The present study explored, whether regorafenib stimulates eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to regorafenib and phosphatidylserine surface abundance, cell volume, $[Ca^{2+}]_i$, reactive oxygen species (ROS) formation, and ceramide abundance determined by 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 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, 1 $CaCl_2$, at 37°C for 48 hours. Where indicated, erythrocytes were exposed for 48 hours to regorafenib (MedChem Express, Princeton, USA). In order to estimate the impact of regorafenib on eryptosis due to high $[Ca^{2+}]_i$, erythrocytes were exposed for 1 hour to a combination of regorafenib and the Ca^{2+} ionophore ionomycin (Merck Millipore, Darmstadt, Germany). To test for an involvement of kinases, erythrocytes were exposed for 48 hours to a combination of regorafenib and p38 kinase inhibitor SB 203580 (Tocris bioscience, Bristol, UK) or protein kinase C inhibitor staurosporine (Sigma Aldrich, Hamburg, Germany).

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 µl cell suspension was washed in Ringer solution containing 5 mM $CaCl_2$ and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 15 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 regorafenib 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".

Intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM $CaCl_2$ and 5 µM Fluo-3/AM. The cells were incubated at 37°C for 30 min. Ca^{2+}-dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Reactive oxygen species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 µl suspension of erythrocytes was washed in Ringer solution and stained with DCFDA (Sigma, Schnelldorf, Germany) in Ringer solution containing DCFDA at a final concentration of 10 µM. Erythrocytes were incubated at 37°C for 30 min in the dark and washed two times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 µl Ringer solution and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

Ceramide abundance

For the determination of ceramide, a monoclonal antibody-based assay was used. To this end, cells were stained for 1 hour at 37°C with 1 µg/ml anti ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. The samples were washed twice with PBS-BSA. The cells were stained for 30 minutes with polyclonal fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. The samples were analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. As a control, secondary antibody alone was used.

Statistics

Data are expressed as arithmetic means ± SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and *t* test as appropriate. n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study explored whether regorafenib stimulates eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Erythrocyte volume was estimated from forward scatter which was determined utilizing flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with regorafenib (0.5 – 5 µg/ml). As illustrated in Fig. 1,

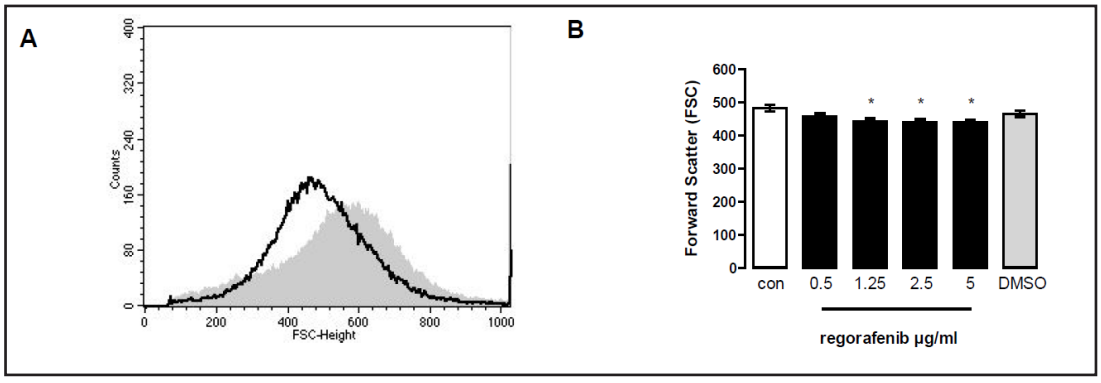

Fig. 1. Effect of regorafenib on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 5 µg/ml regorafenib. B. Arithmetic means ± SEM (n = 20) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) regorafenib (0.5 - 5 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). *($p<0.05$) indicates significant difference from the absence of regorafenib (ANOVA).

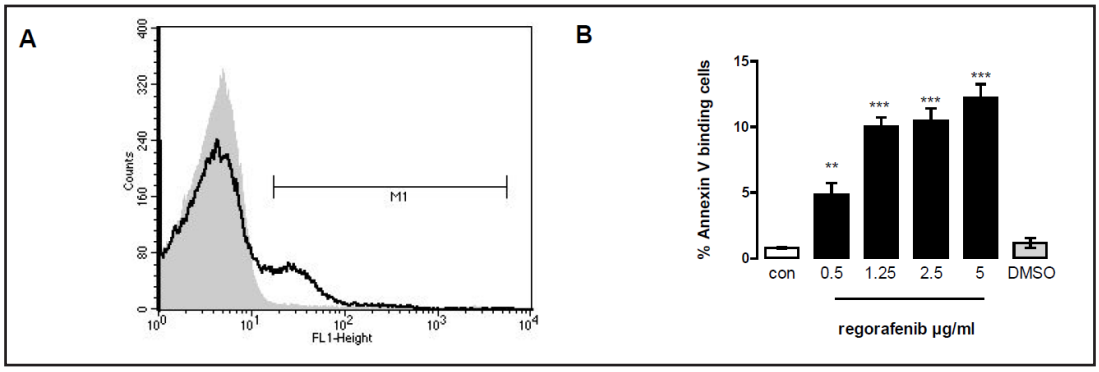

Fig. 2. Effect of regorafenib on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 5 µg/ml regorafenib. B. Arithmetic means ± SEM (n = 20) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) regorafenib (0.5 - 5 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). **(p<0.01),***(p<0.001) indicates significant difference from the absence of regorafenib (ANOVA).

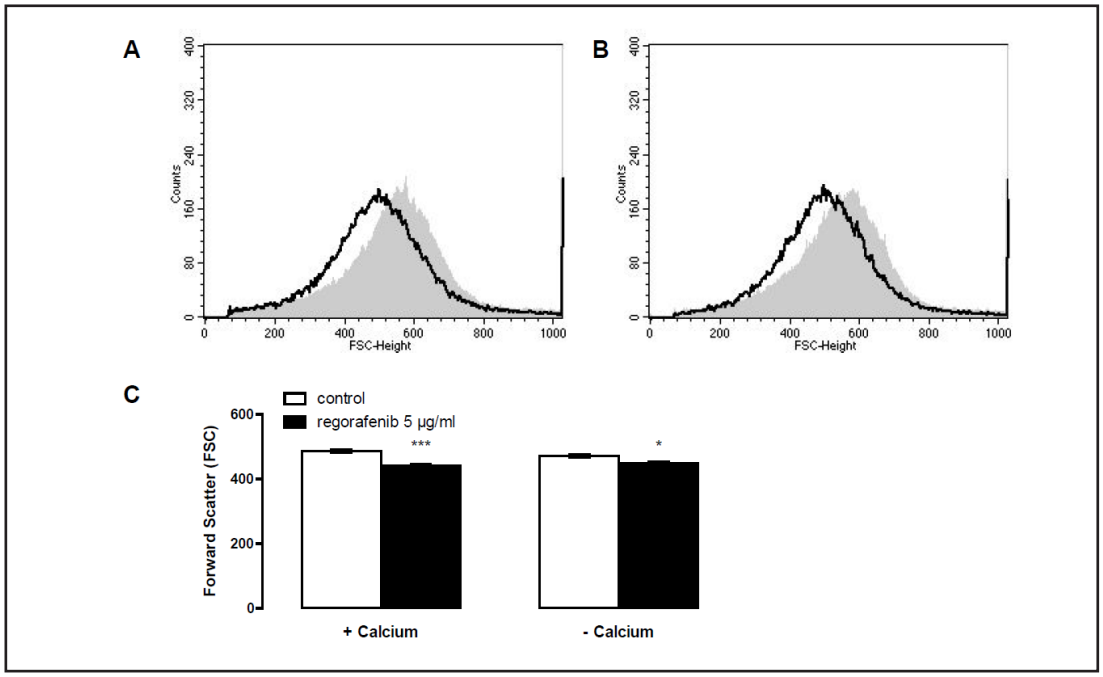

Fig. 3. Ca^{2+} sensitivity of regorafenib -induced erythrocyte shrinkage. A,B. Original histogram of erythrocyte forward scatter following exposure for 48 hours to Ringer solution without (grey area) and with (black line) regorafenib (5 µg/ml) in the presence (A) and absence (B) of extracellular Ca^{2+}. C. Arithmetic means ± SEM (n = 25) of erythrocyte forward scatter after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) regorafenib (5 µg/ml) in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of Ca^{2+}. ***(p<0.001) p,*(p<0.05) indicates significant difference from the absence of regorafenib (ANOVA).

regorafenib decreased erythrocyte forward scatter, an effect reaching statistical significance at 1.25 µg/ml regorafenib concentration.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with regorafenib (0.5 – 5 µg/ml). As shown in Fig. 2, a 48 hours exposure to regorafenib increased the percentage of

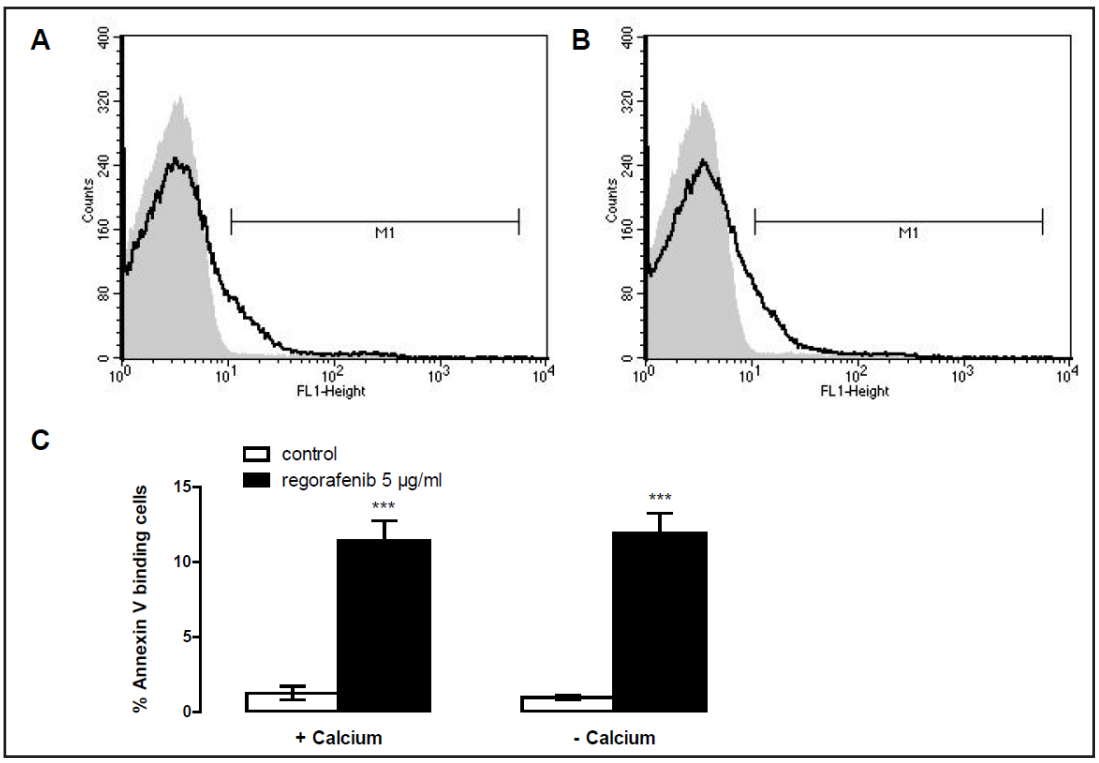

Fig. 4. Ca^{2+} sensitivity of regorafenib -induced phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) regorafenib (5 µg/ml) in the presence (A) and absence (B) of extracellular Ca^{2+}. C. Arithmetic means ± SEM (n = 20) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) regorafenib (5 µg/ml) in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of Ca^{2+}. ***($p<0.001$) indicates significant difference from the absence of regorafenib (ANOVA).

phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 0.5 µg/ml regorafenib.

Fluo3 fluorescence was taken as a measure of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). As a result, following a 48 hours incubation the Fluo3 fluorescence was lower in the presence of 0.5 µg/ml regorafenib (17.1 ± 0.7 a.u., n = 13), 1.25 µg/ml regorafenib (15.7 ± 0.8 a.u., n = 13), 2.5 µg/ml regorafenib (12.6 ± 0.5 a.u., n = 13) and 5 µg/ml regorafenib (12.0 ± 0.5 a.u., n = 13) than in the absence of regorafenib (19.7 ± 0.7 a.u., n = 13). Additional experiments were performed in order to elucidate whether regorafenib affects Fluo3 fluorescence of erythrocytes treated with the Ca^{2+} ionophore ionomycin (1 µM). As a result, 5 µg/ml regorafenib treatment decreased the Fluo3 fluorescence from 21.6 ± 0.7 a.u. (n = 12) to 12.4 ± 0.2, a.u. (n = 12) in the absence of ionomycin and from 65.5 ± 2.5 a.u. (n = 12) to 56.1 ± 2.0 a.u. (n = 12) in the presence of ionomycin.

A next series of experiments explored whether the regorafenib-induced cell shrinkage and translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular Ca^{2+}. To this end, erythrocytes were incubated for 48 hours in the absence or presence of 5 µg/ml regorafenib in the presence or nominal absence of extracellular Ca^{2+}. As illustrated in Fig. 3, removal of extracellular Ca^{2+} did not significantly blunt the effect of regorafenib on forward scatter. Even in the absence of extracellular Ca^{2+}, regorafenib significantly decreased the erythrocyte forward scatter. Thus, regorafenib-induced cell shrinkage may have been in part triggered by mechanisms other than entry of extracellular Ca^{2+}.

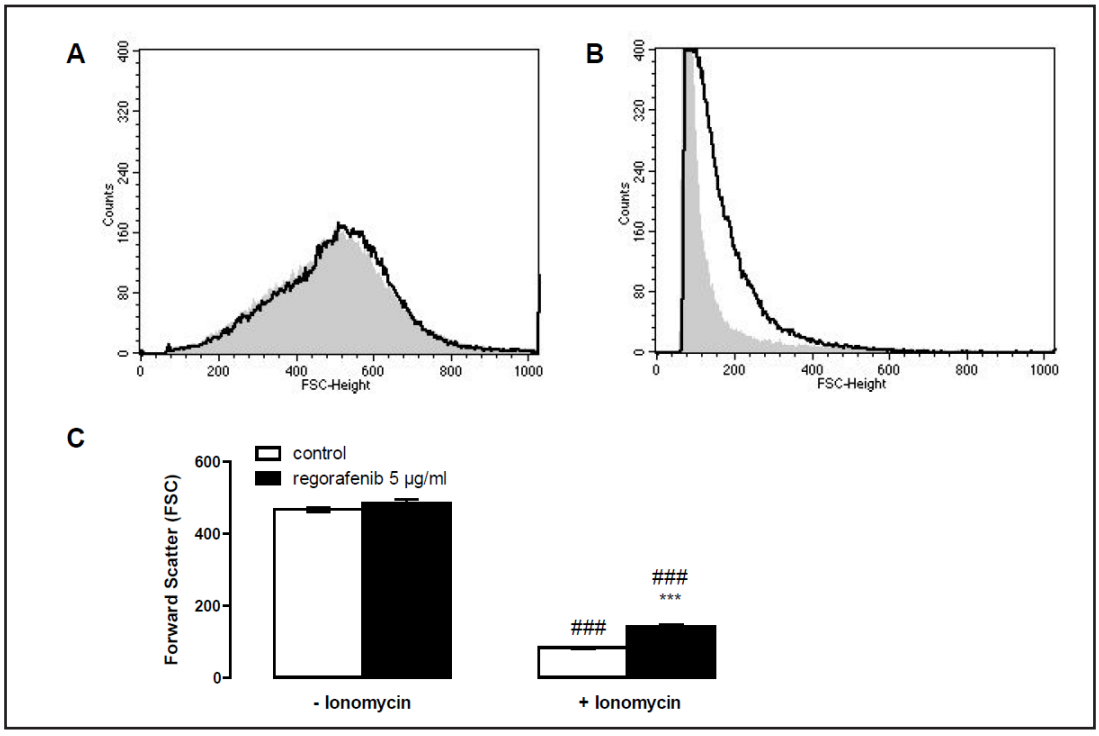

Fig. 5. Effect of regorafenib and Ca^{2+} ionophore ionomycin on erythrocyte forward scatter. A,B. Original histogram of erythrocyte forward scatter following exposure for 1 h to Ringer solution without (grey area) and with (black line) regorafenib (5 µg/ml) in the absence (A) and presence (B) of ionomycin (1µM). C. Arithmetic means ± SEM (n = 5) of erythrocyte forward scatter after a 1 h treatment with Ringer solution without (white bars) or with (black bars) regorafenib (5 µg/ml) in the absence (left bars, -ionomycin) and presence (right bars, +ionomycin) of ionomycin (1 µM). ***(p<0.001) indicates significant difference from the absence of regorafenib, ###(p<0.001) indicates significant difference from the absence of ionomycin (ANOVA).

Similar observations were made with annexin-V-binding. Even in the absence of extracellular Ca^{2+}, regorafenib significantly increased the percentage of annexin-V-binding erythrocytes (Fig. 4). Thus, regorafenib-induced cell membrane scrambling was in large part triggered by mechanisms other than entry of extracellular Ca^{2+}.

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). As a result, the DCFDA fluorescence was similar following exposure to 5 µg/ml regorafenib (15.2 ± 0.3, a.u., n = 9) and in the absence of regorafenib (15.3 ± 0.9, a.u., n = 9). Thus, regorafenib did not appreciably induce oxidative stress.

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As a result, the ceramide abundance was similar following exposure to 5 µg/ml regorafenib (15.0 ± 0.3 a.u., n = 4) and in the absence of regorafenib (14.0 ± 0.5 a.u., n = 4). Thus, regorafenib did not appreciably induce ceramide abundance.

To explore, whether the effects of regorafenib involved kinase activity, the influence of regorafenib on annexin-V-binding and forward scatter was tested in the presence of p38 kinase inhibitor SB 203580 or protein kinase C inhibitor staurosporine. Regorafenib (5 µg/ml) increased phosphatidylserine exposure to similar values in the absence (from 1.3 ± 0.1 % to 15.5 ± 1.6 %, n = 10) and in the presence (from 1.2 ± 0.1 % to 17.1 ± 1.6 %, n = 10) of SB 203580 (2 µM). Moreover, regorafenib (5 µg/ml) increased phosphatidylserine exposure to similar values in the absence (from 1.3 ± 0.1 % to 15.5 ± 1.6 %, n = 10) and in the presence (from 1.5 ± 0.2 % to 16.3 ± 1.8 %, n = 10) of staurosporine (1 µM).

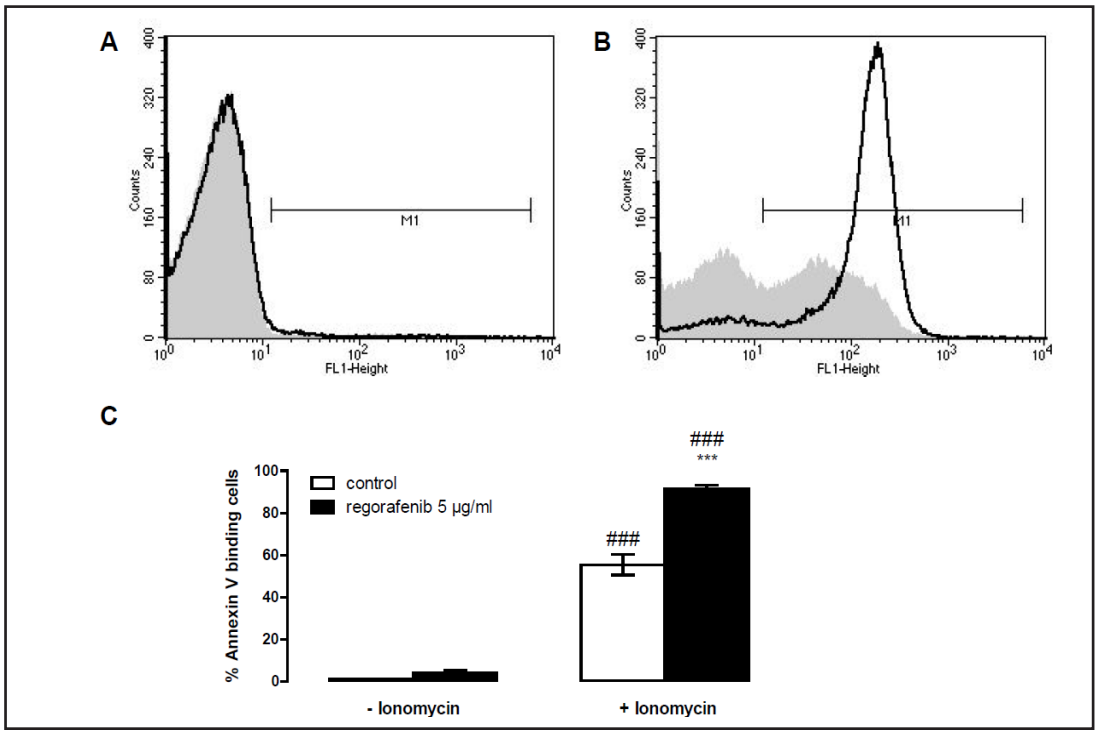

Fig. 6. Effect of regorafenib and Ca^{2+} ionophore ionomycin on phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 1 h to Ringer solution without (grey area) and with (black line) regorafenib (5 µg/ml) in the absence (A) and presence (B) of ionomycin (1 µM). C. Arithmetic means ± SEM (n = 5) of annexin-V-binding of erythrocytes after a 1 h treatment with Ringer solution without (white bars) or with (black bars) regorafenib (5 µg/ml) in the absence (left bars, -ionomycin) and presence (right bars, +ionomycin) of ionomycin (1 µM). ***($p<0.001$) indicates significant difference from the absence of regorafenib, ###($p<0.001$) indicates significant difference from the absence of ionomycin (ANOVA).

In order to test, whether regorafenib is effective downstream of Ca^{2+}, the cytosolic Ca^{2+} activity was increased by Ca^{2+} ionophore ionomycin (1 µM). As illustrated in Fig. 5, ionomycin treatment was followed by a similar decrease of forward scatter in the absence and presence of regorafenib. As shown in Fig. 6, regorafenib significantly increased the percentage of annexin-V-binding erythrocytes even in the presence of ionomycin. Thus, regorafenib-induced cell membrane scrambling was in large part triggered by mechanisms downstream of Ca^{2+}.

Discussion

The present observations uncover a novel effect of regorafenib, i.e. the triggering of suicidal erythrocyte death or eryptosis. Exposure of human erythrocytes to regorafenib results in cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface.

The effect of regorafenib on cell membrane scrambling was not dependent on Ca^{2+} entry from the extracellular space, as removal of extracellular Ca^{2+} did not significantly modify regorafenib induced eryptosis. Moreover, regorafenib augmented cell membrane scrambling in erythrocytes loaded with Ca^{2+} by treatment with the Ca^{2+} ionophore ionomycin. Thus, regorafenib apparently sensitized the cell to the scrambling effect of Ca^{2+} and was effective downstream of Ca^{2+}. Cells could be sensitized for the scrambling effect of Ca^{2+} by ceramide [39]. However, regorafenib triggered cell membrane scrambling and cell shrinkage without enhancing the ceramide abundance. Moreover, regorafenib triggered eryptosis without inducing

oxidative stress. Moreover, the effect of regorafenib did not require the activity of p38 kinase or of staurosporine sensitive kinases such as protein kinase C. The regorafenib induced cell membrane scrambling was thus independent from increase of cytosolic Ca^{2+} activity, oxidative stress, ceramide formation or activity of p38 kinase and staurosporine sensitive kinases, the major triggers of eryptosis [39].

The effect of regorafenib on cell shrinkage was again seemingly not dependent on Ca^{2+} entry from the extracellular space. As Ca^{2+} has been added shortly before measurements, the observations do, however, not completely rule out involvement of Ca^{2+}. Interestingly, regorafenib significantly blunted the effect of the Ca^{2+} ionophore ionomycin on cell shrinkage. The ionomycin induced cell shrinkage is due to increase of $[Ca^{2+}]_i$ with subsequent activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water. The underlying mechanism of regorafenib-induced erythrocyte swelling in the presence of ionomycin remained elusive. Potential mechanisms include impairment of Na^+/K^+ ATPase activity with decrease of cellular K^+ and thus interference with Ca^{2+} induced hyperpolarization.

The purpose of eryptosis is the clearance of defective erythrocytes from circulating blood prior to hemolysis [39]. Hemolysis otherwise leads to release of hemoglobin, which passes the renal glomerular filter, precipitates in the acidic lumen of renal tubules, occludes nephrons and thus may lead to renal failure [75]. Eryptosis is further triggered by infection of erythrocytes with the malaria pathogen *Plasmodium*, which imposes oxidative stress on the infected host erythrocyte thus activating Ca^{2+}-permeable erythrocyte cation channels [39, 76]. Accelerated eryptosis fosters the clearance of infected erythrtocytes from circulating blood thus decreasing parasitemia. The clinical course of malaria is thus favourably influenced by accelerated eryptosis, as in sickle-cell trait, beta-thalassemia-trait, Hb-C and G6PD-deficiency [39, 77-79] or in eryptosis stimulating conditions, such as iron deficiency [80] and treatment with lead [80], chlorpromazine [81] or NO synthase inhibitors [81]. Possibly, regorafenib similarly enhances the susceptibility of *Plasmodium* infected erythrocytes to eryptosis.

As phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood, stimulation of eryptosis may lead to anemia as soon as the loss of erythrocytes surpasses the formation of new erythrocytes by erythropoiesis [39]. The regorafenib concentrations required for the stimulation of eryptosis are well in the range of concentrations encountered in the plasma of patients [82]. Thus, the observed stimulation of eryptosis may well explain the anemia following regorafenib treatment. The effect of regorafenib treatment on eryptosis may be enhanced in clinical conditions with accelerated eryptosis, such as dehydration [83], hyperphosphatemia [84], chronic kidney disease (CKD) [85-88], hemolytic-uremic syndrome [89], diabetes [90], hepatic failure [91], malignancy [39], sepsis [92], sickle-cell disease [39], beta-thalassemia [39], Hb-C and G6PD-deficiency [39], as well as Wilsons disease [93].

Phosphatidylserine exposing erythrocytes may further adhere to the vascular wall [94], stimulate blood clotting and trigger thrombosis [95-97], thus impairing microcirculation [41, 95, 98-101].

Conclusion

Regorafenib triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect apparently independent from Ca^{2+} entry, oxidative stress and ceramide.

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Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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