Stimulation of Suicidal Erythrocyte Death by Ceritinib-Treatment of Human Erythrocytes

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

Abstract
Background/Aims: The anaplastic lymphoma kinase (ALK) inhibitor ceritinib is utilized for the treatment of ALK positive non-small cell lung carcinoma. Side effects of the drug include decrease of blood hemoglobin concentration. Possible causes of anemia include stimulation of suicidal erythrocyte death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Signaling of eryptosis includes increase of cytosolic Ca$^{2+}$ activity ([Ca$^{2+}$]), oxidative stress, ceramide, staurosporine sensitive protein kinase C, SB203580 sensitive p38 kinase, D4476 sensitive casein kinase 1, and zVAD sensitive caspases. The present study explored, whether ceritinib induces eryptosis and, if so, to shed light on the cellular mechanisms involved.

Methods: Phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, [Ca$^{2+}$] from Fluo3-fluorescence, ROS formation from DCFDA dependent fluorescence, and ceramide abundance utilizing specific antibodies.

Results: A 48 hours exposure of human erythrocytes to ceritinib (1 µg/ml) significantly increased the percentage of annexin-V-binding cells, significantly decreased forward scatter, significantly increased Fluo3-fluorescence, but did not significantly modify DCFDA fluorescence or ceramide abundance. The effect of ceritinib on annexin-V-binding was significantly blunted but not abolished by removal of extracellular Ca$^{2+}$, by the kinase inhibitors staurosporine (1 µM), SB203580 (2 µM) and D4476 (10 µM), as well as by caspase inhibitor zVAD (10 µM).

Conclusions: Ceritinib triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect at least in part due to Ca$^{2+}$ entry, as well as activation of kinases and caspases.
Introduction

The anaplastic lymphoma (tyrosine) kinase (ALK) inhibitor ceritinib is utilized for the treatment of ALK positive non-small cell lung carcinoma [1-15]. The most common laboratory abnormality following ceritinib treatment is a decrease of blood hemoglobin concentration [2]. Potential causes of anemia include stimulation of suicidal erythrocyte death or eryptosis [16], which leads to rapid clearance of affected erythrocytes from circulating blood and thus to anemia [16]. Hallmarks of eryptosis include cell shrinkage [17] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [16]. Eryptosis may be triggered by increase of cytosolic Ca^{2+} activity ([Ca^{2+}]_i) [16], ceramide [18], oxidative stress [16], and energy depletion [16]. Eryptosis may or may not involve caspases [16, 19, 20]. Kinases able to stimulate eryptosis include casein kinase 1α [21], Janus-activated kinase JAK3 [22], protein kinase C [23], and p38 kinase [24]. Kinases protecting against eryptosis include AMP activated kinase AMPK [25], cGMP-dependent protein kinase [26], PAK2 kinase [27], and sorafenib/sunitinib sensitive kinases [28, 29]. Eryptosis is triggered by multiple xenobiotics [16, 30-71].

The present study tested, whether ceritinib triggers eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to ceritinib and phosphatidylserine surface abundance, cell volume, [Ca^{2+}]_i, 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 ceritinib (Selleckchem, Munich, Germany). In order to estimate the impact of Ca^{2+} entry, erythrocytes were exposed to ceritinib in the presence and absence of extracellular Ca^{2+}. To test for an involvement of kinases, erythrocytes were exposed for 48 hours to a combination of ceritinib and protein kinase C inhibitor staurosporine (Enzo Life Sciences, Lörrach, Germany), p38 kinase inhibitor SB 203580 (Tocris bioscience, Bristol, UK) or casein kinase inhibitor D4476 (Tocris Bioscience, Bristol, UK). To test for an involvement of caspases, erythrocytes were exposed for 48 hours to a combination of ceritinib and pan-caspase inhibitor zVAD (Enzo Life Sciences, Lörrach, Germany).

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 µl cell suspension of erythrocytes was centrifuged at 1600 rpm for 3 min and, after discarding the supernatant, the erythrocytes were 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 ceritinib 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 then 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 and washed once in Ringer solution containing 5 mM CaCl_2. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer solution. Then, Ca^{2+}-dependent 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. Afterwards, the geomean of the Ca\(^{2+}\) dependent fluorescence was determined.

**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 then stained with DCFDA (Sigma Aldrich, Hamburg, 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 two times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 µl Ringer solution, and ROS-dependent fluorescence intensity was measured in FL-1 at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD). Subsequently, the geomean of the ROS-dependent fluorescence was determined.

**Ceramide abundance**

To determine the ceramide abundance at the erythrocyte surface, a monoclonal antibody was used. After incubation, cells were stained for 1 h at 37°C with 1 µg/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in phosphate-buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA) at a dilution of 1:10. After two washing steps with PBS-BSA, cells were stained for 30 min with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (BD Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. Samples were then analyzed by flow cytometric analysis in FL-1 at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Finally, the geomean of the ceramide dependent fluorescence was determined.

**Hemolysis**

Following incubation, the erythrocyte suspension was centrifuged for 3 min at 1600 rpm, 4°C, and the supernatant harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration in 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 ± 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**

As ceritinib treatment has been shown to trigger anemia, the present study explored the possibility that ceritinib stimulates eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. Prior to flow cytometry, the erythrocytes were incubated for 48 hours in Ringer solution without or with ceritinib (0.5 – 1 µg/ml). As shown in Fig. 1, a 48 hours exposure to ceritinib increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 0.75 µg/ml ceritinib.

Erythrocyte volume was estimated from forward scatter which was determined utilizing flow cytometry. Again, erythrocytes were incubated for 48 hours in Ringer solution without or with ceritinib (0.5 – 1 µg/ml) prior to measurements. As illustrated in Fig. 2, ceritinib decreased erythrocyte forward scatter, an effect statistically significant at 1 µg/ml ceritinib concentration.
Hemoglobin concentration in the supernatant was taken as a measure of hemolysis. The erythrocytes were again incubated 48 hours prior to measurements in Ringer solution without or with ceritinib (0.5 – 1 µg/ml). As shown in Fig. 3, a 48 hours exposure to ceritinib increased the percentage of hemolytic erythrocytes, an effect statistically significant at 1 µg/ml ceritinib.

Fluo3 fluorescence was taken as a measure of cytosolic Ca^{2+} activity ([Ca^{2+}]_{i}). The erythrocytes were again incubated for 48 hours prior to measurements in Ringer solution either without or with ceritinib (0.5 – 1 µg/ml). As illustrated in Fig. 4, ceritinib increased Fluo3 fluorescence, an effect again reaching statistical significance at 1 µg/ml ceritinib concentration.

A further series of experiments explored whether the ceritinib-induced translocation of phosphatidylserine required entry of extracellular Ca^{2+}. To this end, erythrocytes were...
incubated for 48 hours in the absence or presence of 1 µg/ml ceritinib in the presence or nominal absence of extracellular Ca$^{2+}$. As illustrated in Fig. 5, removal of extracellular Ca$^{2+}$ significantly blunted the effect of ceritinib on annexin-V-binding. However, even in the absence of extracellular Ca$^{2+}$, ceritinib significantly increased the percentage of annexin-V-binding erythrocytes (Fig. 5). Thus, the ceritinib-induced cell membrane scrambling was in part triggered by mechanisms other than entry of extracellular Ca$^{2+}$.

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) were thus quantified utilizing 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) following a 48 hours incubation in Ringer solution without or with ceritinib (1 µg/ml). As a result, the DCFDA fluorescence was similar following exposure to 1 µg/ml ceritinib (22.06 ± 1.16 a.u., n = 9) and in the absence of ceritinib (20.71 ± 0.61 a.u., n = 9). Thus, ceritinib 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 after a 48 hours incubation of the erythrocytes in Ringer solution without or with ceritinib (1 µg/ml). The ceramide abundance was similar following exposure to 1 µg/ml ceritinib (20.56 ± 2.29 a.u., n = 10) and in the
absence of ceritinib (19.34 ± 1.94 a.u., n = 10). Accordingly, ceritinib did not appreciably increase ceramide abundance.

To explore, whether the effects of ceritinib involved kinase activity, the influence of ceritinib on annexin-V-binding was tested in the absence and presence of kinase inhibitors. As illustrated in Fig. 6, addition of staurosporine (1 µM) significantly blunted the effect of ceritinib on annexin-V-binding. However, even in the presence of staurosporine, ceritinib significantly increased the percentage of annexin-V-binding erythrocytes (Fig. 6). Thus, the ceritinib-induced cell membrane scrambling was in part dependent on staurosporine sensitive kinases. As shown in Fig. 7, addition of p38 kinase inhibitor SB203580 (2 µM) significantly blunted the effect of ceritinib on annexin-V-binding. Again, ceritinib significantly increased annexin-V-binding of erythrocytes in the absence and presence of SB203580 (2 µM) (ANOVA).
increased the percentage of annexin-V-binding erythrocytes even in the presence of SB203580 (Fig. 7). Thus, the ceritinib-induced cell membrane scrambling was partially but not fully dependent on p38 kinase. As illustrated in Fig. 8, addition of specific casein kinase 1 inhibitor D4476 (10 µM) significantly blunted the effect of ceritinib on annexin-V-binding. However, ceritinib significantly increased the percentage of annexin-V-binding erythrocytes even in the presence of D4476 (10 µM). ***(p<0.001) indicates significant difference from the absence of ceritinib, #(p<0.05) indicates significant difference from the absence of D4476 (10 µM) (ANOVA).

A final series of experiments explored the putative involvement of caspases. As shown in Fig. 9, addition of pan-caspase inhibitor zVAD (10 µM) significantly blunted the effect of ceritinib on annexin-V-binding. However, even in the presence of zVAD, ceritinib significantly

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Fig. 8. D4476 sensitivity of ceritinib-induced phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) ceritinib (1 µg/ml) in the absence (A) and presence (B) of D4476 (10 µM). C. Arithmetic means ± SEM (n = 15) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) ceritinib (1 µg/ml) in the absence (left bars) and presence (right bars) of D4476 (10 µM). ***(p<0.001) indicates significant difference from the absence of ceritinib, #(p<0.05) indicates significant difference from the absence of D4476 (10 µM) (ANOVA).

Fig. 9. zVAD sensitivity of ceritinib-induced phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) ceritinib (1 µg/ml) in the absence (A) and presence (B) of zVAD (10 µM). C. Arithmetic means ± SEM (n = 11) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) ceritinib (1 µg/ml) in the absence (left bars) and presence (right bars) of zVAD (10 µM). *(p<0.05), ***(p<0.001) indicates significant difference from the absence of ceritinib, #(p<0.05) indicates significant difference from the absence of zVAD (10 µM) (ANOVA).
increased the percentage of annexin-V-binding erythrocytes (Fig. 9). Thus, the ceritinib-induced cell membrane scrambling was in part dependent on caspase activity.

Discussion

The present observations disclose a novel effect of ceritinib, i.e. the stimulation of suicidal erythrocyte death or eryptosis. Exposure of human erythrocytes to ceritinib results in cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentration required for the stimulation of eryptosis is well in the range of concentrations (up to 1.4 µg/ml) encountered in plasma of patients [1]. Stimulation of eryptosis could thus well contribute to or even account for the ceritinib-induced anemia [2]. It must be kept in mind that the sensitivity to triggers of eryptosis is enhanced in several clinical conditions including iron deficiency [16], dehydration [72], hyperphosphatemia [73], chronic kidney disease (CKD) [74-77], hemolytic-uremic syndrome [78], diabetes [79], hepatic failure [63], malignancy [16], sepsis [80], sickle-cell disease [16], beta-thalassemia [16], Hb-C and G6PD-deficiency [16], as well as Wilsons disease [81].

The effect of ceritinib on cell membrane scrambling was paralleled by increase of cytosolic Ca²⁺ activity ([Ca²⁺]) and required in part Ca²⁺ entry from the extracellular space. Removal of extracellular Ca²⁺ significantly blunted the ceritinib-induced phosphatidylserine translocation. However, even in the nominal absence of extracellular Ca²⁺ ceritinib significantly stimulated cell membrane scrambling, an observation pointing to the involvement of additional mechanisms. Cells could be sensitized for the scrambling effect of Ca²⁺ by ceramide [16]. However, ceritinib triggered cell membrane scrambling without enhancing ceramide abundance. Moreover, ceritinib triggered eryptosis without inducing oxidative stress. Instead, the effect of ceritinib was sensitive to protein kinase C inhibitor staurosporine, p38 kinase inhibitor SB203580 and casein kinase inhibitor D4476. All those kinases have previously been shown to trigger eryptosis [16]. Moreover, the ceritinib-induced eryptosis was sensitive to caspases, which may, dependent on the trigger, participate in the stimulation of eryptosis [16]. Known stimulators of caspases in erythrocytes include oxidative stress [16, 19, 20], which, however, appears to be absent following ceritinib treatment. Erythrocytes lack mitochondria and are thus unable to activate caspases through the mitochondrial pathway. The mechanism underlying ceritinib-induced caspase activation thus remained elusive.

The effect of ceritinib on cell membrane scrambling may similarly involve Ca²⁺ entry from the extracellular space, which is expected to increase [Ca²⁺] with subsequent activation of Ca²⁺ sensitive K⁺ channels, K⁺ exit, cell membrane hyperpolarization, Cl⁻ exit and thus cellular loss of KCl with water.

Ceritinib tended to increase hemolysis at 0.5 and 0.75 µg/ml, an effect, however, not reaching statistical significance. At 1 µg/ml, ceritinib triggered a robust, statistically significant hemolysis. Unlike eryptosis [16], hemolysis leads to release of hemoglobin, which passes the renal glomerular filter, precipitates in the acidic lumen of renal tubules, occludes nephrons and thus causes renal failure [82].

As phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood, stimulation of eryptosis is followed by anemia as soon as the loss of erythrocytes outcasts the formation of new erythrocytes by erythropoiesis [16]. Anemia could further result from stimulated hemolysis and/or enhanced apoptosis of progenitor cells [83, 84]. Phosphatidylserine exposing erythrocytes may further compromise microcirculation [18, 85-89] due to adherence to the vascular wall [90], as well as stimulation of blood clotting and thrombosis [85, 91, 92].

In conclusion, ceritinib triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect in part due to Ca²⁺ entry, activation of staurosporine, SB203580 and D4476 sensitive kinases as well as caspases. The present observations may point to therapeutic options in the prevention of ceritinib-induced anemia. For instance, eryptosis were expected to be inhibited by skepinone, an anti-eryptotic p38 kinase inhibitor, which is effective in vivo [93].
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Disclosure Statement

All authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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