Original Paper

Stimulation of Eryptosis by Afatinib

Abdulla Al Mamun Bhuyan^a Florian Lang^{b,c}^aDepartment of Internal Medicine III, ^bDepartment of Vegetative and Clinical Physiology, Eberhard-Karls-University of Tuebingen, Tuebingen, ^cDepartment of Molecular Medicine II, Medical Faculty, Heinrich Heine University, Düsseldorf, Germany

Key Words

Afatinib • Eryptosis • Phosphatidylserine • Calcium • Oxidative stress • Ceramide

Abstract

Background/Aims: The epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitor afatinib is primarily utilized for the treatment of non-small cell lung carcinoma. The drug is at least partially effective by triggering suicidal tumor cell death. Side effects of afatinib treatment include anemia. At least in theory, afatinib induced anemia could be secondary to stimulation of suicidal erythrocyte death or eryptosis, characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Signaling potentially stimulating eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), induction of oxidative stress, and increase of ceramide abundance. The present study explored, whether afatinib induces eryptosis and, if so, whether its effect involves Ca^{2+} entry, oxidative stress, and/or ceramide. **Methods:** Flow cytometry was employed to quantify phosphatidylserine exposure at the cell surface from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, reactive oxygen species (ROS) abundance from DCFDA dependent fluorescence, and ceramide abundance utilizing specific antibodies. **Results:** A 48 hours exposure of human erythrocytes to afatinib ($\geq 4 \mu\text{g/ml}$) significantly increased the percentage of annexin-V-binding cells and significantly decreased forward scatter. Afatinib significantly increased Fluo3-fluorescence, DCFDA fluorescence and ceramide abundance. The effect of afatinib on annexin-V-binding and forward scatter was significantly blunted by removal of extracellular Ca^{2+} . **Conclusions:** Afatinib triggers phospholipid scrambling of the erythrocyte cell membrane, an effect at least in part due to Ca^{2+} entry, oxidative stress, and ceramide.

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Published by S. Karger AG, Basel

Introduction

Afatinib, a second generation epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitor [1-17], is primarily utilized for the treatment of non small cell lung carcinoma [1, 3, 5, 7, 9-14, 16-40]. Side effects of afatinib include skin rash [6], xerosis [6], paronychia [6], stomatitis [6, 41], diarrhea [6, 41], heart failure [42], neutropenia [41], leukopenia [41],

and anemia [41, 43]. Afatinib is in part effective by inducing apoptosis [44-57], an effect involving oxidative stress [46].

At least in theory, the anemia following afatinib treatment could be secondary to suicidal death of erythrocytes or eryptosis [58-60]. Hallmarks of eryptosis are cell shrinkage [61] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [58]. Cellular mechanisms participating in the orchestration of eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [58], ceramide [62], caspases [58, 63, 64], G-protein Galphai2 [65], casein kinase 1 α [58], Janus-activated kinase JAK3 [58], protein kinase C [58], and p38 kinase [58]. Eryptosis is suppressed by AMP activated kinase AMPK [58], cGMP-dependent protein kinase [58], mitogen and stress activated kinase MSK1/2 [66], PAK2 kinase [58] and sorafenib/sunitinib sensitive kinases [58]. Eryptosis could be triggered by cell stress, such as hyperosmotic shock [58], oxidative stress [58], energy depletion [58], radiation [67, 68], or exposure to a wide variety of substances [58, 66, 69-112]. Several substances inhibit eryptosis [113-116]. Enhanced eryptosis is observed in diverse clinical conditions including iron deficiency [58], vitamin D excess [117], chronic kidney disease (CKD) [118-123], hemolytic-uremic syndrome [124], autoimmune hemolytic anemia [125], diabetes [126], hypertension and dyslipidemia [127], hepatic failure [128], malignancy [129-131], arteritis [132], systemic lupus erythematosus [133], sepsis [134, 135], malaria [58, 136, 137], sickle-cell disease [58], beta-thalassemia [58], Hb-C and G6PD-deficiency [58], Wilsons disease [134], as well as advanced age [138]. Eryptosis further increases following storage for transfusion [67, 68, 83, 139] and is enhanced in erythrocytes from newborns exposed to oxidative stress [58, 140].

The present study explored, whether exposure of erythrocytes drawn from healthy volunteers to afatinib influences cell membrane scrambling and cell volume. Additional experiments were performed to shed light on the signaling involved.

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 afatinib (MedChem Express, Princeton, USA). In order to estimate the impact of Ca^{2+} entry on afatinib induced phosphatidylserine exposure, ROS and ceramide formation, erythrocytes were exposed to afatinib in the absence and presence of extracellular Ca^{2+} . To explore the participation of Akt/PKB signaling pathway, erythrocytes were exposed for 48 hours to a combination of afatinib and Akt1/2 kinase inhibitor A6730 (58nM) (Sigma Aldrich 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 , stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) and incubated 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) [141]. 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 afatinib 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 μ M 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. 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 μ l 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 μ M. 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 μ l Ringer solution and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD). Subsequently, the geomean of the DCFDA dependent fluorescence was determined.

Ceramide abundance

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

Hemolysis

For the determination of hemolysis, the samples were centrifuged (3 min. at 1600 rpm in 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 $\pm$ 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*

Fig. 1. Effect of afatinib 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 8 μ g/ml afatinib. B. Arithmetic means $\pm$ SEM (*n* = 15) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (Control) or with (black bars) afatinib (2 - 8 μ g/ml). For comparison, the effect of the solvent DMSO is shown (grey bar).

***(*p* < 0.001) indicates significant difference from the absence of afatinib (ANOVA).

Fig. 2. Effect of Afatinib on erythrocyte forward scatter. A. Original histograms of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 8 $\mu\text{g/ml}$ Afatinib. B. Arithmetic means $\pm$ SEM ($n = 15$) of the erythrocyte forward scatter (FSC) following incubation for

48 hours to Ringer solution without (white bar) or with (black bars) Afatinib (2 - 8 $\mu\text{g/ml}$) or solvent DMSO alone (grey bar). ***($p < 0.001$) indicates significant difference from the absence of afatinib (ANOVA).

Fig. 3. Effect of Afatinib on Fluo3 fluorescence. A. Original histograms of Fluo3 fluorescence reflecting cytosolic Ca^{2+} activity of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 8 $\mu\text{g/ml}$ Afatinib. B. Arithmetic means $\pm$ SEM ($n = 15$) of the Fluo3 fluo-

rescence in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Afatinib (2 - 8 $\mu\text{g/ml}$) or solvent DMSO alone (grey bar). **($p < 0.01$), ***($p < 0.001$) indicates significant difference from the absence of afatinib (ANOVA).

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 afatinib stimulates eryptosis, the suicidal erythrocyte death. The most important hallmark of eryptosis is phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface. Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding to phosphatidylserine, as determined by flow cytometry. Annexin-V-binding was analysed following an incubation of the erythrocytes for 48 hours in Ringer solution without or with afatinib (2 - 8 $\mu\text{g/ml}$). As illustrated in Fig. 1, a 48 hours exposure to afatinib increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 4 $\mu\text{g/ml}$ afatinib.

A second hallmark of eryptosis is erythrocyte shrinkage. As a measure of erythrocyte volume, forward scatter was determined utilizing flow cytometry. The measurements were performed after incubation for 48 hours in Ringer solution without or with afatinib (2 - 8 $\mu\text{g/ml}$). As shown in Fig. 2, afatinib decreased the erythrocyte forward scatter, an effect reaching statistical significance at 4 $\mu\text{g/ml}$ afatinib.

Eryptosis could be triggered by increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Fluo3 fluorescence was employed in order to quantify $[\text{Ca}^{2+}]_i$. The erythrocytes were analyzed after a 48 hours incubation in Ringer solution without or with afatinib (2 - 8 $\mu\text{g/ml}$). As illustrated in Fig. 3, a 48 hours exposure to afatinib increased the Fluo3 fluorescence, an effect reaching

Fig. 4. Ca^{2+} sensitivity of afatinib-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) afatinib (8 $\mu\text{g}/\text{ml}$) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means $\pm$ SEM ($n = 15$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) afatinib (8 $\mu\text{g}/\text{ml}$) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . *($p < 0.05$), ***($p < 0.001$) indicates significant difference from the absence of afatinib, ###($p < 0.001$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

Fig. 5. Ca^{2+} sensitivity of afatinib-induced decrease of forward scatter. A,B. Original histograms of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) afatinib (8 $\mu\text{g}/\text{ml}$) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means $\pm$ SEM ($n = 15$) of forward scatter of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) afatinib (8 $\mu\text{g}/\text{ml}$) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . *($p < 0.05$), ***($p < 0.001$) indicates significant difference from the absence of afatinib, ###($p < 0.001$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

Fig. 6. Effect of afatinib on reactive oxygen species. A. Original histogram of DCFDA fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 8 $\mu\text{g}/\text{ml}$ afatinib. B. Arithmetic means $\pm$ SEM ($n = 13$) of DCFDA fluorescence in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bar) afatinib (8 $\mu\text{g}/\text{ml}$). ***($p < 0.001$) indicates significant difference from the absence of afatinib (unpaired t test).

statistical significance at 4 $\mu\text{g}/\text{ml}$ afatinib. In this series of experiments, afatinib (8 $\mu\text{g}/\text{ml}$) significantly decreased the forward scatter from 533.00 ± 16.61 to 428.90 ± 13.82 ($n = 6$).

Fig. 7. Ca^{2+} sensitivity of afatinib-induced reactive oxygen species. A,B. Original histograms of DCFDA fluorescence of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) afatinib (8 $\mu\text{g}/\text{ml}$) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means $\pm$ SEM (n = 8) of DCFDA fluorescence of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) afatinib (8 $\mu\text{g}/\text{ml}$) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . ***($p < 0.001$) indicates significant difference from the absence of afatinib, ##($p < 0.01$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

Fig. 8. Effect of afatinib on ceramide abundance. A. Original histogram of ceramide abundance at the surface of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 8 $\mu\text{g}/\text{ml}$ afatinib. B. Arithmetic means $\pm$ SEM (n = 5) of ceramide abundance at the surface of erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bar) afatinib (8 $\mu\text{g}/\text{ml}$). ***($p < 0.01$) indicates significant difference from the absence of afatinib (unpaired t test).

In order to test whether afatinib-induced translocation of phosphatidylserine to the outside required entry of extracellular Ca^{2+} , erythrocytes were incubated for 48 hours in the absence or presence of 8 $\mu\text{g}/\text{ml}$ afatinib in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 4, removal of extracellular Ca^{2+} significantly blunted the effect of afatinib on annexin-V-binding. However, even in the absence of extracellular Ca^{2+} , afatinib significantly increased the percentage of annexin-V-binding erythrocytes. Thus, the afatinib-induced cell membrane scrambling was in part but not fully triggered by entry of extracellular Ca^{2+} .

Similar observations were made on erythrocyte cell volume. As illustrated in Fig. 5, removal of extracellular Ca^{2+} significantly blunted the effect of afatinib

Fig. 9. Effect of afatinib on hemolysis. Arithmetic means $\pm$ SEM (n = 5) of the percentage hemolytic erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bar) afatinib (2-8 $\mu\text{g}/\text{ml}$). ***($p < 0.001$) indicates significant difference from the absence of afatinib (unpaired t test).

on forward scatter. However, even in the absence of extracellular Ca^{2+} , afatinib significantly decreased the erythrocyte forward scatter. Thus, the afatinib-induced erythrocyte shrinkage was in part but not fully triggered by entry of extracellular Ca^{2+} .

An additional series of experiments explored the theoretical possibility that afatinib was in part effective by modifying Akt activity. To this end experiments were performed in the absence and presence of Akt inhibitor A6730 (58 nM). As a result, the increase of the percentage of phosphatidylserine exposing erythrocytes following a 48 hours exposure to afatinib (8 $\mu\text{g}/\text{ml}$) was similar in the absence (from 2.46 ± 0.52 to 23.64 ± 2.19 %, $n = 5$) and presence (from 4.07 ± 0.47 to 25.26 ± 6.41 %, $n = 5$) of Akt inhibitor A6730 (58 nM).

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As illustrated in Fig. 6, a 48 hours exposure to afatinib (8 $\mu\text{g}/\text{ml}$) increased the DCFDA fluorescence of erythrocytes, observations pointing to afatinib induced oxidative stress. In this series of experiments, afatinib (8 $\mu\text{g}/\text{ml}$) significantly decreased the forward scatter from 484.30 ± 10.21 to 436.00 ± 18.60 ($n = 8$). The increase of DCFDA fluorescence was significantly more pronounced in the absence than in the presence of extracellular Ca^{2+} (Fig. 7).

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was quantified utilizing specific antibodies. As illustrated in Fig. 8, a 48 hours exposure to afatinib (8 $\mu\text{g}/\text{ml}$) increased the ceramide abundance at the erythrocyte surface. In this series of experiments, afatinib (8 $\mu\text{g}/\text{ml}$) significantly decreased the forward scatter from 483.50 ± 8.78 to 424.60 ± 15.38 ($n = 10$). The increase of ceramide abundance following a 48 hours exposure to afatinib (8 $\mu\text{g}/\text{ml}$) was similar in the presence (from 9.59 ± 0.24 to 12.84 ± 0.86 a.u., $n = 5$) and absence (from 11.64 ± 0.81 to 14.08 ± 0.43 a.u., $n = 5$) of extracellular Ca^{2+} . The increase of ceramide abundance following a 48 hours exposure to afatinib (8 $\mu\text{g}/\text{ml}$) was further similar in the absence (from 9.41 ± 0.29 to 11.63 ± 0.29 a.u., $n = 5$) and presence (from 9.33 ± 0.26 to 11.79 ± 0.39 a.u., $n = 5$) of Akt inhibitor A6730 (58 nM).

A final series of experiments addressed the effect of afatinib on hemolysis, as evident from the haemoglobin concentration in the supernatant. As illustrated in Fig. 9, a 48 hours exposure to afatinib at 8 $\mu\text{g}/\text{ml}$, but not at lower concentrations increased the percentage of hemolytic erythrocytes.

Discussion

The present observations reveal that afatinib stimulates cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface, and triggers erythrocyte shrinkage, apparent from a decrease of forward scatter. Cell membrane scrambling and cell shrinkage are hallmarks of eryptosis, the suicidal erythrocyte death. The afatinib concentrations required for the stimulation of eryptosis are within the range of concentrations encountered in the plasma of patients [43]. The stimulation of eryptosis may thus well explain the anemia following afatinib treatment [31].

The effect of afatinib on cell membrane scrambling was paralleled by an increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) and was in large part dependent on Ca^{2+} entry from the extracellular space. Removal of extracellular Ca^{2+} significantly blunted the afatinib induced eryptosis. However, afatinib triggered cell membrane scrambling even in the nominal absence of extracellular Ca^{2+} , an observation pointing to the involvement of additional mechanisms contributing to afatinib induced cell membrane scrambling. Cells could be sensitized for the scrambling effect of Ca^{2+} by ceramide [58]. Afatinib treatment indeed increased the ceramide abundance at the erythrocyte surface. Moreover, afatinib significantly enhanced the abundance of reactive oxygen species, a further known stimulator of eryptosis [58]. Accordingly, afatinib is in part effective by inducing oxidative stress.

The effect of afatinib on cell membrane scrambling was paralleled by erythrocyte shrinkage. The effect is significantly blunted by Ca^{2+} removal and is presumably in large part due to increase of $[\text{Ca}^{2+}]_i$ with subsequent activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water.

Phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood and stimulation of eryptosis may lead to anemia as soon as the loss of erythrocytes surpasses the formation of new erythrocytes by erythropoiesis [58]. Stimulation of eryptosis may further compromise microcirculation [62, 142-146] as phosphatidylserine exposing erythrocytes adhere to the vascular wall [147], stimulate blood clotting and trigger thrombosis [142, 148, 149]. On the other hand, eryptosis may precede and thus prevent hemolysis [58] with release of hemoglobin, which could pass the renal glomerular filter, precipitate in the acidic lumen of renal tubules, occlude nephrons and may thus lead to renal failure [150].

In conclusion, afatinib triggers erythrocyte cell membrane scrambling, an effect in part due to Ca^{2+} entry, oxidative stress as well as ceramide.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Lejla Subasic. The study was supported by the Deutsche Forschungsgemeinschaft and the open access publishing fund of the University Tübingen.

Disclosure Statement

The authors declare to have no conflict of interests.

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