Efavirenz Induced Suicidal Death of Human Erythrocytes

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Key Words
Phosphatidylserine • Cell volume • Eryptosis • Oxidative stress • Calcium • p38 kinase • Casein kinase

Abstract
Background/Aims: The reverse transcriptase inhibitor efavirenz utilized for the treatment of human immunodeficiency virus (HIV)-1 infection, triggers suicidal cell death or apoptosis, an effect in part due to interference with mitochondrial potential. Side effects of efavirenz include anemia. Causes of anemia include accelerated clearance of circulating erythrocytes. Even though lacking mitochondria, erythrocytes may enter suicidal erythrocyte death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Triggers of eryptosis include Ca\textsuperscript{2+} entry and increase of cytosolic Ca\textsuperscript{2+} activity ([Ca\textsuperscript{2+}]\textsubscript{i}), oxidative stress, ceramide, as well as activation of p38 kinase, casein kinase 1\alpha and/or cyclooxygenase. The present study explored, whether and how efavirenz induces eryptosis. \textbf{Methods:} Phosphatidylserine exposure at the cell surface was estimated from annexin V binding, cell volume from forward scatter, [Ca\textsuperscript{2+}]\textsubscript{i}, from Fluo3-fluorescence, ROS formation from DCFDA dependent fluorescence, and ceramide abundance utilizing selective antibodies. \textbf{Results:} A 48 hours exposure of human erythrocytes to efavirenz (≥ 2 µg/ml) significantly increased the percentage of annexin-V-binding cells, significantly decreased forward scatter (2 µg/ml), significantly increased Fluo3-fluorescence (≥ 2 µg/ml), but did not significantly modify DCFDA fluorescence or ceramide abundance. The effect of efavirenz on annexin-V-binding was significantly blunted, but not abolished by removal of extracellular Ca\textsuperscript{2+}. The effect of efavirenz on annexin-V-binding was further significantly blunted by p38 kinase inhibitor SB203580 (2 µM) and casein kinase 1\alpha inhibitor D4476 (10 µM), but not by cyclooxygenase inhibitor aspirin (50 µM). \textbf{Conclusions:} Efavirenz triggers cell shrinkage and phosphatidylserine translocation to the erythrocyte surface, an effect in part due to stimulation of Ca\textsuperscript{2+} entry as well as activation of p38 kinase and casein kinase 1\alpha.
Introduction

The non-nucleoside reverse transcriptase inhibitor efavirenz is used in the treatment of human immunodeficiency virus (HIV)-1 infection [1-25]. Side effects of efavirenz include diverse neuropsychiatric disorders, such as neurocognitive impairment, nightmares, dizziness, insomnia, nervousness and lack of concentration, mania, depression, suicidal thoughts, psychosis, hallucinations [1, 10, 26-34], cutaneous adverse drug reactions [35], hypersensitivity reaction [36, 37], hepatotoxicity [37], dyslipidemia [21, 38] and anemia [39, 40].

Efavirenz has been shown to trigger apoptosis [41-45], an effect in part due to interference with mitochondrial potential and function [41, 43, 45].

Potential causes of anemia include stimulation of eryptosis [46], the suicidal death of erythrocytes characterized by cell shrinkage [47] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [46]. Erythrocytes lack mitochondria and nuclei, key organelles in the machinery of apoptosis, but may enter eryptosis following opening of Ca\(^{2+}\)-permeable unselective cation channels with subsequent Ca\(^{2+}\) entry, increase of cytosolic Ca\(^{2+}\) activity \([\text{Ca}^{2+}]_i\), and Ca\(^{2+}\)-sensitive stimulation of cell membrane scrambling and cell shrinkage [46]. Further stimulators of eryptosis include oxidative stress [46], ceramide [48], energy depletion [46], activated caspases [46, 49, 50], stimulated activity of casein kinase 1α [51], Janus-activated kinase JAK3 [52], protein kinase C, p38 kinase [53], as well as pharmacological and/or genetic knockout of AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase and sorafenib/sunitinib sensitive kinases [46]. Eryptosis is triggered by diverse xenobiotics [46, 54-78]. Susceptibility to triggers of eryptosis is enhanced in several clinical conditions, such as dehydration [67], hyperphosphatemia [77], chronic kidney disease (CKD) [59, 79-81], hemolytic-uremic syndrome [82], diabetes [83], hepatic failure [84], malignancy [85], sepsis [86], Sickle-cell disease [87], beta-thalassemia [87], Hb-C and G6PD-deficiency [46, 87], as well as Wilsons disease [88].

The present study explored whether and how efavirenz induces eryptosis. To this end, human erythrocytes from healthy volunteers were treated with efavirenz and phosphatidylserine surface abundance, cell volume, [Ca\(^{2+}\)]_i, reactive oxygen species 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 x 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. The strong dilution of the erythrocytes aimed to blunt triggering of eryptosis by release of eryptosis inducing substances from neighbouring erythrocytes. Where indicated, erythrocytes were exposed to efavirenz (MedChem Express, Princeton, USA) at the indicated concentrations.

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 in FL-1 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 efavirenz 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. 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.

**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, 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 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).

**Determination of ceramide formation**

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation, 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. Subsequently, the cells were stained for 30 minutes with polyclonal fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. The samples were resuspended in 200 µl PBS-BSA and then analyzed in FL-1 by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

**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 the putative effect of efavirenz on eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface. Annexin-V-binding determined by flow cytometry was taken as a measure of phosphatidylserine abundance at the erythrocyte surface. Prior to the measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with efavirenz (1 - 4 µg/ml). As illustrated in Fig. 1, a 48 hours exposure to efavirenz increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 2 µg/ml efavirenz.

Forward scatter was determined by flow cytometry as an estimate of erythrocyte volume. Again, the measurements were made after a 48 hours incubation in Ringer solution without or with efavirenz (1 - 4 µg/ml). As illustrated in Fig. 2, the exposure to efavirenz decreased erythrocyte forward scatter, an effect reaching statistical significance at 2 µg/ml efavirenz.

Fluo3 fluorescence was taken as measure of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]). As illustrated in Fig. 3, a 48 hours exposure to efavirenz increased the Fluo3 fluorescence, an effect reaching statistical significance at 2 µg/ml efavirenz.
In order to explore whether the efavirenz-induced translocation of phosphatidylserine required entry of extracellular Ca\(^{2+}\), erythrocytes were incubated for 48 hours in the absence or presence of 4 µg/ml efavirenz 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 efavirenz on annexin-V-binding. However, even in the absence of extracellular Ca\(^{2+}\), efavirenz significantly increased...
the percentage of annexin-V-binding erythrocytes. Thus, efavirenz-induced cell membrane scrambling was partially but not fully triggered by entry of extracellular Ca$^{2+}$.

In order to test whether the efavirenz-induced translocation of phosphatidylserine required the activation of p38 kinase, erythrocytes were exposed to 4 µg/ml efavirenz for 48 hours in the absence or nominal presence of p38 kinase inhibitor SB203580 (2 µM). As demonstrated in Fig. 5, the phosphatidylserine exposure following efavirenz treatment was significantly blunted in the presence of p38 kinase inhibitor SB203580 (2 µM).

Further experiments explored whether the efavirenz-induced annexin V binding required the activation of casein kinase 1α. To this end, erythrocytes were exposed to 4 µg/ml efavirenz for 48 hours in the absence or nominal presence of casein kinase 1α inhibitor D4476 (10 µM). As illustrated in Fig. 6, the annexin V binding following efavirenz treatment was significantly diminished in the presence of casein kinase inhibitor D4476 (10 µM).

In order to test whether formation of cyclooxygenase product PGE$_2$ is involved in the suicidal erythrocyte death triggered by efavirenz, erythrocytes were exposed to 4 µg/ml efavirenz for 48 hours in the absence or presence of cyclooxygenase inhibitor aspirin (50 µM). As a result, the percentage of annexin V binding was similar in the presence (34.3 ± 3.6 a.u., n=10) and in the absence (33.4 ± 2.7 a.u., n=10) of 50 µM aspirin.

In order to explore the involvement of erythrocyte necroptosis [89, 90], an inhibitor of RIP1, necrostatin-1 (10 µM) and an inhibitor of MLKL, necrosulfonamide (1 µM), have been tested. Erythrocytes were exposed to 4 µg/ml efavirenz for 48 hours in the absence or presence of necrostatin-1 (10 µM) or necrosulfonamide (1 µM). As a result, annexin-V-binding tended to be higher in the presence (31.5 ± 8.7 a.u., n=5) than in the absence (23.8 ± 3.1 a.u., n=5) of 10 µM necrostatin-1, a difference, however, not reaching statistical significance. Following a 48 hours incubation with 4 µg/ml efavirenz, annexin V binding was similar in the presence (41.4 ± 5.4 a.u., n=6) and in the absence (43.8 ± 5.6 a.u., n=6) of 1 µM necrosulfonamide.
Ca\(^{2+}\) entry and subsequent eryptosis is stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). As a result, following a 48 hours incubation, the DCFDA fluorescence was similar following exposure to 4 µg/ml efavirenz (15.9 ± 1.5 a.u., n = 7) and in the absence of efavirenz (17.3 ± 1.0 a.u., n = 7). Thus, efavirenz did not significantly modify the abundance of reactive oxygen species.

Eryptosis is further stimulated by ceramide. The ceramide abundance was thus quantified utilizing specific antibodies. As a result, following a 48 hours incubation, the ceramide abundance was similar following exposure to 4 µg/ml efavirenz (16.0 ± 0.7 a.u., n = 7) and in the absence of efavirenz (17.6 ± 1.3 a.u., n = 7). Thus, efavirenz did not significantly modify the ceramide abundance.

**Discussion**

The present observations reveal a novel effect of efavirenz, i.e. the triggering of suicidal erythrocyte death or eryptosis. It is among the more powerful stimulators of eryptosis [46]. Treatment of human erythrocytes to efavirenz is followed by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Phosphatidylserine translocating erythrocytes are rapidly cleared from circulating blood [46]. To the extent that the eryptosis is not matched by a similar increase of erythropoiesis, stimulation of eryptosis may well lead to anemia [46]. The efavirenz concentrations required for the effect of efavirenz on eryptosis may be reached in vivo [91]. Thus, the observed eryptosis may contribute to the anemia following efavirenz treatment. Moreover, uncovering the underlying mechanisms may open opportunities to counteract efavirenz induced anemia.

The stimulation of cell membrane scrambling by efavirenz was in part due to stimulation of Ca\(^{2+}\) entry. Thus, the effect of efavirenz on cell membrane scrambling was paralleled by an increase of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_i\)), and was significantly blunted, albeit not abolished, by removal of extracellular Ca\(^{2+}\). An increase of [Ca\(^{2+}\)]\(_i\) activates an undefined scramblase thus triggering cell membrane scrambling [46]. An increase of [Ca\(^{2+}\)]\(_i\) further activates Ca\(^{2+}\) sensitive K\(^+\) channels with subsequent cell shrinkage due to K\(^+\) exit, cell membrane hyperpolarization, Cl\(^-\) exit and thus cellular loss of KCl with water [47].

Pharmacological evidence suggests that the full effect of efavirenz requires activity of casein kinase and p38 kinase. According to DCFDA fluorescence, efavirenz treatment did not appreciably increase the abundance of reactive oxygen species and according to specific antibodies, efavirenz treatment did not significantly modify the ceramide abundance at the erythrocyte surface. Oxidative stress and ceramide are well known causes of eryptosis [46].

The physiological function of eryptosis is the clearance of defective erythrocytes from circulating blood [46]. The timely clearance of defective erythrocytes prevents hemolysis with release of hemoglobin, which would pass the renal glomerular filter, precipitate in the acidic lumen of renal tubules and thus occlude nephrons [92]. In malaria, the pathogen *Plasmodium* activates Ca\(^{2+}\)-permeable host erythrocyte cation channels with subsequent eryptosis and clearance of the infected host erythrocyte from circulating blood [46, 93]. Sickle-cell trait, beta-thalassemia-trait, Hb-C and G6PD-deficiency foster eryptosis, accelerate the clearance of infected erythrocytes, decrease parasitemia and thus protect against a severe course of malaria [46, 94-96]. Similarly, iron deficiency [97], and exposure to lead [97], chlorpromazine [98] or NO synthase inhibitors [98] foster eryptosis thus decreasing parasitemia. In view of the present observations, the theoretical possibility must be considered that efavirenz may similarly enhance the susceptibility of *Plasmodium* infected erythrocytes to eryptosis.

Phosphatidylserine exposing erythrocytes may, however, adhere to the vascular wall [99], stimulate blood clotting and trigger thrombosis [100-102]. Thus, stimulation of eryptosis may compromise microcirculation [48, 100, 103-106].

In conclusion, efavirenz triggers cell membrane scrambling in erythrocytes, an effect paralleled by and in part due to increase of cytosolic Ca\(^{2+}\) activity.

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Disclosure Statement

The authors declare that they have nothing to disclose.

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