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Original Paper

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^{2+} entry and increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), oxidative stress, ceramide, as well as activation of p38 kinase, casein kinase 1α and/or cyclooxygenase. The present study explored, whether and how efavirenz induces eryptosis. ***Methods:*** 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 selective antibodies. ***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^{2+}. The effect of efavirenz on annexin-V-binding was further significantly blunted by p38 kinase inhibitor SB203580 (2 μM) and casein kinase 1α inhibitor D4476 (10 μM), but not by cyclooxygenase inhibitor aspirin (50 μM). ***Conclusions:*** Efavirenz triggers cell shrinkage and phosphatidylserine translocation to the erythrocyte surface, an effect in part due to stimulation of Ca^{2+} entry as well as activation of p38 kinase and casein kinase 1α.

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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 ($[Ca^{2+}]_i$), 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 ($[Ca^{2+}]_i$). 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.

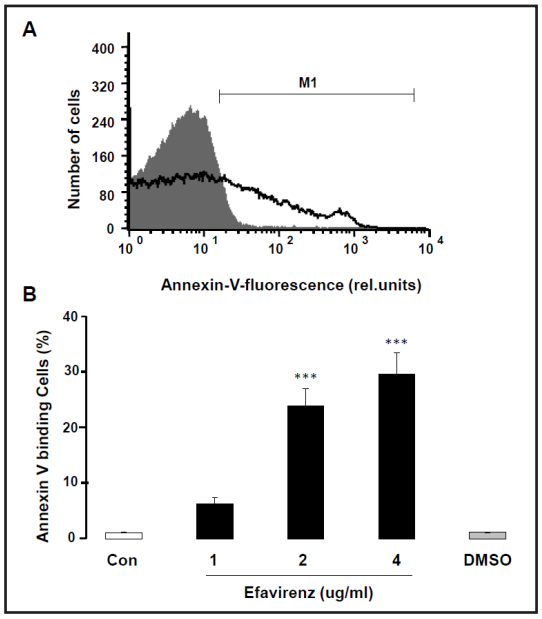


Fig. 1. Effect of efavirenz on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 4 µg/ml efavirenz. B. Arithmetic means ± SEM (n = 19) of erythrocyte annexin-V-binding (black bars) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of efavirenz (1 – 4 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). ***(p<0.001) indicates significant difference from the absence of efavirenz (ANOVA).

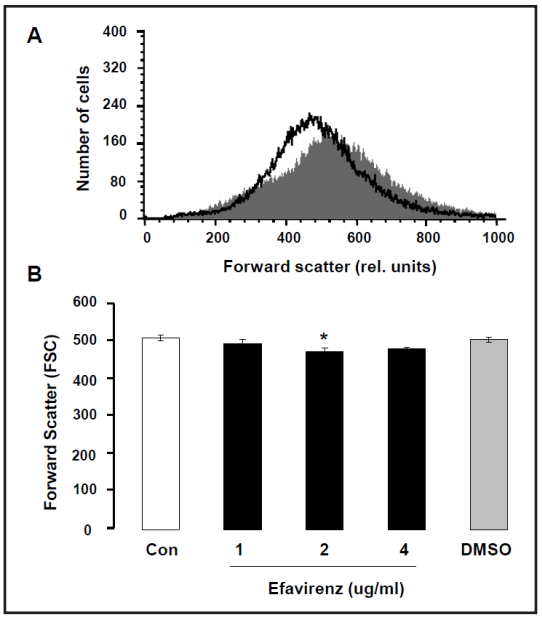
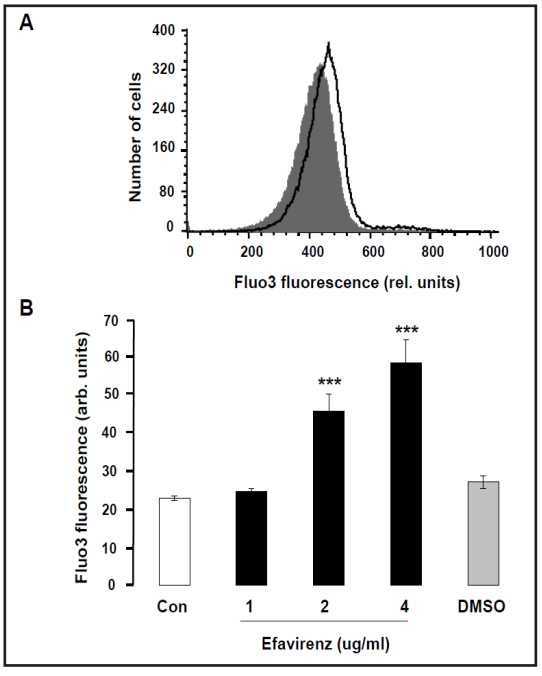


Fig. 2. Effect of efavirenz on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 4 µg/ml efavirenz. B. Arithmetic means ± SEM (n = 19) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of efavirenz (1 - 4 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). *(p<0.05) indicates significant difference from the absence of efavirenz (ANOVA).

Fig. 3. Effect of efavirenz on erythrocyte Ca^{2+} activity. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 4 µg/ml efavirenz. B. Arithmetic means ± SEM (n = 19) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) presence of efavirenz (1 – 4 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). ***(p<0.001) indicates significant difference from the absence of efavirenz (ANOVA).



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.

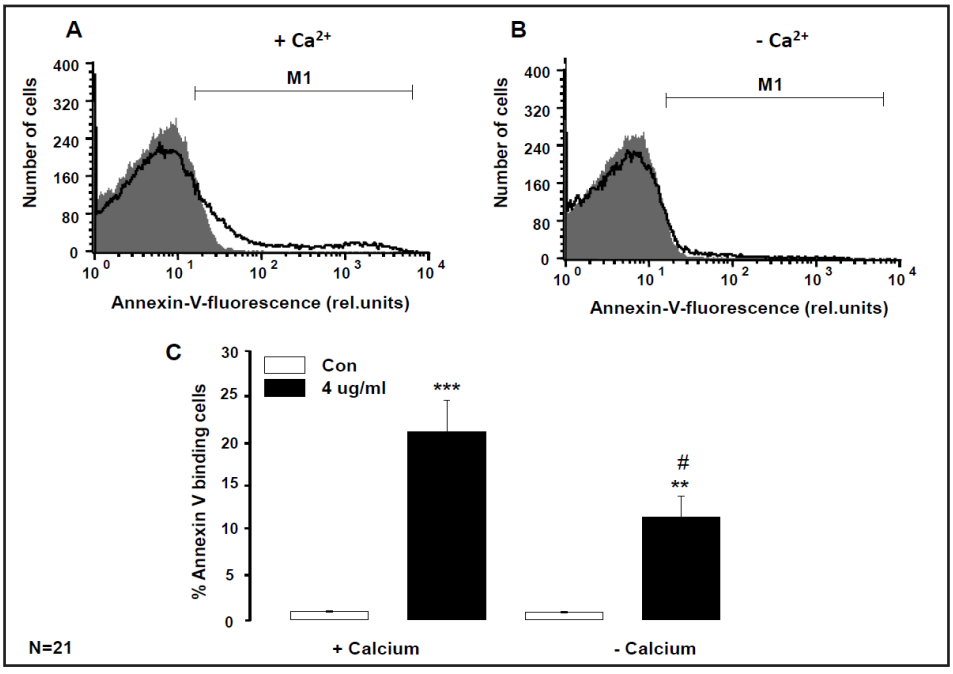


Fig. 4. Ca^{2+} sensitivity of efavirenz-induced phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) presence of 4 µg/ml efavirenz in the presence (A) and absence (B) of extracellular Ca^{2+}. C. Arithmetic means ± SEM (n = 21) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) 4 µg/ml efavirenz in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of Ca^{2+}. **(p<0.01), ***(p<0.001) indicate significant difference from the absence of efavirenz, #(p<0.05) indicates significant difference from the presence of Ca^{2+} (ANOVA).

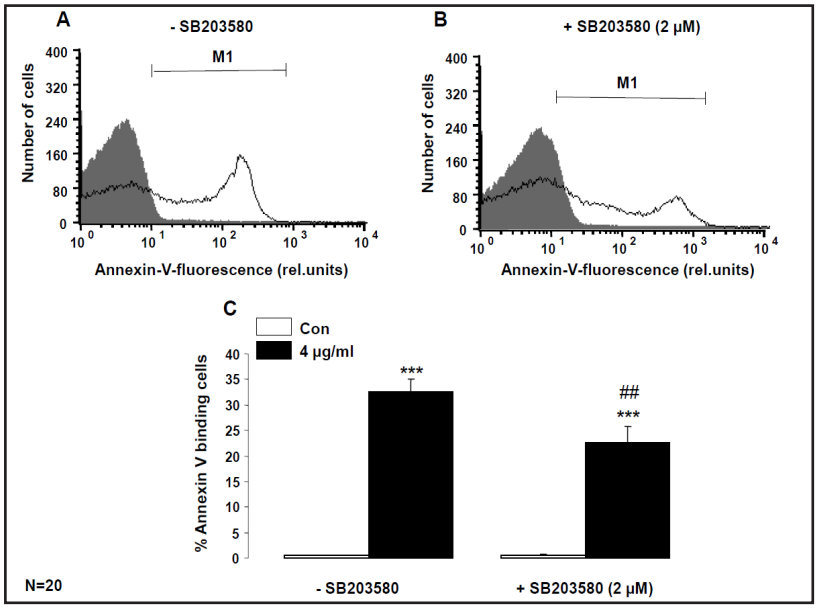


Fig. 5. Effect of efavirenz-induced phosphatidylserine exposure in the presence and absence of p38 kinase inhibitor SB203580 (2 µM). A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) presence of 4 µg/ml efavirenz in the absence (A) and presence (B) of p38 kinase inhibitor SB203580 (2 µM). 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) 4 µg/ml efavirenz in the absence (left bars, -SB203580) and presence (right bars, +SB203580) of SB203580 (2 µM). ***(p<0.001) indicates significant difference from the absence of efavirenz, ##(p<0.01) indicates significant difference from the absence of SB203580 (2 µM) (ANOVA).

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

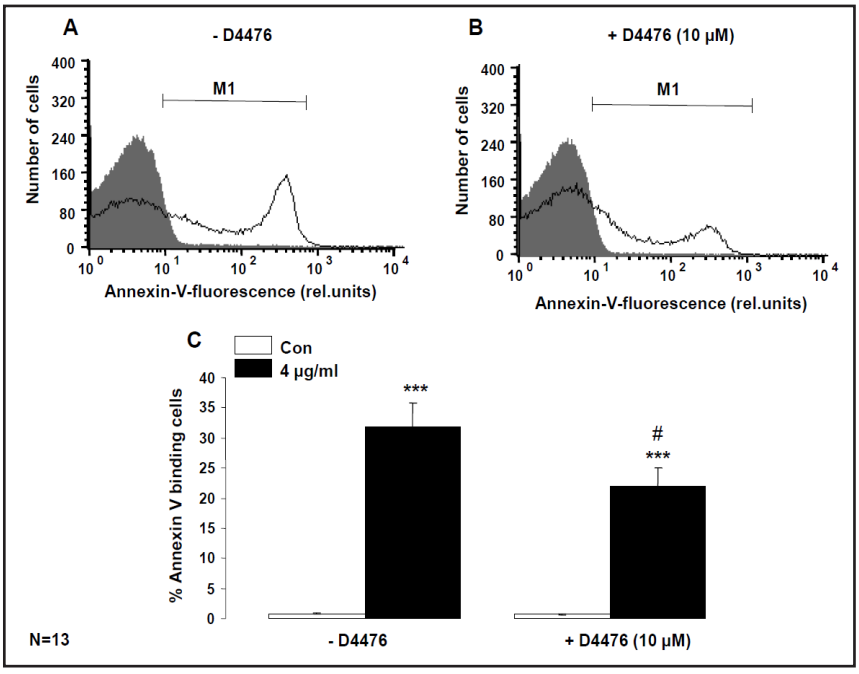


Fig. 6. Effect of efavirenz-induced phosphatidylserine exposure in the presence and absence of casein kinase 1α inhibitor D4476 (10 µM). A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) presence of 4 µg/ml efavirenz in the absence (A) and presence (B) of casein kinase 1α inhibitor D4476 (10 µM). C. Arithmetic means ± SEM (n=13) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) 4 µg/ml efavirenz in the absence (left bars, -D4476) and presence (right bars, +D4476) of D4476 (10 µM). ***(p<0.001) indicates significant difference from the absence of efavirenz, #(p<0.05) indicates significant difference from the absence of D4476 (10 µM) (ANOVA).

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 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 of (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 illdefined 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 compromize 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.

References

1 Apostolova N, Funes HA, Blas-Garcia A, Galindo MJ, Alvarez A, Esplugues JV: Efavirenz and the CNS: what we already know and questions that need to be answered. J Antimicrob Chemother 2015;10.1093/jac/dkv183
2 Bartlett JA, Chen SS, Quinn JB: Comparative efficacy of nucleoside/nucleotide reverse transcriptase inhibitors in combination with efavirenz: results of a systematic overview. HIV Clin Trials 2007;8:221-226.
3 Best BM, Goicoechea M: Efavirenz--still first-line king? Expert Opin Drug Metab Toxicol 2008;4:965-972.
4 Castillo-Mancilla JR, Campbell TB: Comparative effectiveness of efavirenz-based antiretroviral regimens in resource-limited settings. J Comp Eff Res 2012;1:157-170.
5 Colic A, Alessandrini M, Pepper MS: Pharmacogenetics of CYP2B6, CYP2A6 and UGT2B7 in HIV treatment in African populations: focus on efavirenz and nevirapine. Drug Metab Rev 2015;47:111-123.
6 Dahri K, Ensom MH: Efavirenz and nevirapine in HIV-1 infection : is there a role for clinical pharmacokinetic monitoring? Clin Pharmacokinet 2007;46:109-132.
7 Deeks ED, Perry CM: Efavirenz/emtricitabine/tenofovir disoproxil fumarate single-tablet regimen (Atripla(R)): a review of its use in the management of HIV infection. Drugs 2010;70:2315-2338.
8 Ford N, Calmy A, Mofenson L: Safety of efavirenz in the first trimester of pregnancy: an updated systematic review and meta-analysis. AIDS 2011;25:2301-2304.
9 Ford N, Mofenson L, Kranzer K, Medu L, Frigati L, Mills EJ, Calmy A: Safety of efavirenz in first-trimester of pregnancy: a systematic review and meta-analysis of outcomes from observational cohorts. AIDS 2010;24:1461-1470.
10 Ford N, Shubber Z, Pozniak A, Vitoria M, Doherty M, Kirby C, Calmy A: Comparative Safety and Neuropsychiatric Adverse Events Associated With Efavirenz Use in First-Line Antiretroviral Therapy: A Systematic Review and Meta-Analysis of Randomized Trials. J Acquir Immune Defic Syndr 2015;69:422-429.
11 Goicoechea M, Best B: Efavirenz/emtricitabine/tenofovir disoproxil fumarate fixed-dose combination: first-line therapy for all? Expert Opin Pharmacother 2007;8:371-382.
12 Jiang HY, Zhang MN, Chen HJ, Yang Y, Deng M, Ruan B: Nevirapine versus efavirenz for patients co-infected with HIV and tuberculosis: a systematic review and meta-analysis. Int J Infect Dis 2014;25:130-135.
13 King J, Aberg JA: Clinical impact of patient population differences and genomic variation in efavirenz therapy. AIDS 2008;22:1709-1717.
14 Kryst J, Kawalec P, Pilc A: Efavirenz-Based Regimens in Antiretroviral-Naive HIV-Infected Patients: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. PLoS One 2015;10:e0124279.
15 Larru B, Eby J, Lowenthal ED: Antiretroviral treatment in HIV-1 infected pediatric patients: focus on efavirenz. Pediatric Health Med Ther 2014;5:29-42.
16 Maggiolo F: Efavirenz. Expert Opin Pharmacother 2007;8:1137-1145.
17 Maggiolo F: Efavirenz: a decade of clinical experience in the treatment of HIV. J Antimicrob Chemother 2009;64:910-928.
18 Mbuagbaw LC, Irlam JH, Spaulding A, Rutherford GW, Siegfried N: Efavirenz or nevirapine in three-drug combination therapy with two nucleoside-reverse transcriptase inhibitors for initial treatment of HIV infection in antiretroviral-naive individuals. Cochrane Database Syst Rev 2010;10.1002/14651858.CD004246.pub3CD004246.

19 Pillay P, Ford N, Shubber Z, Ferrand RA: Outcomes for efavirenz versus nevirapine-containing regimens for treatment of HIV-1 infection: a systematic review and meta-analysis. PLoS One 2013;8:e68995.
20 Raffi F, Pozniak AL, Wainberg MA: Has the time come to abandon efavirenz for first-line antiretroviral therapy? J Antimicrob Chemother 2014;69:1742-1747.
21 Rahman AP, Eaton SA, Nguyen ST, Bain AM, Payne KD, Bedimo R, Busti AJ: Safety and efficacy of simvastatin for the treatment of dyslipidemia in human immunodeficiency virus-infected patients receiving efavirenz-based highly active antiretroviral therapy. Pharmacotherapy 2008;28:913-919.
22 Rakhmanina NY, van den Anker JN: Efavirenz in the therapy of HIV infection. Expert Opin Drug Metab Toxicol 2010;6:95-103.
23 Saberi P, Caswell N, Amodio-Groton M, Alpert P: Pharmacy-refill measure of adherence to efavirenz can predict maintenance of HIV viral suppression. AIDS Care 2008;20:741-745.
24 Torno MS, Witt MD, Saitoh A, Fletcher CV: Successful use of reduced-dose efavirenz in a patient with human immunodeficiency virus infection: case report and review of the literature. Pharmacotherapy 2008;28:782-787.
25 Vrouenraets SM, Wit FW, van Tongeren J, Lange JM: Efavirenz: a review. Expert Opin Pharmacother 2007;8:851-871.
26 Arendt G, de Nocker D, von Giesen HJ, Nolting T: Neuropsychiatric side effects of efavirenz therapy. Expert Opin Drug Saf 2007;6:147-154.
27 Cavalcante GI, Capistrano VL, Cavalcante FS, Vasconcelos SM, Macedo DS, Sousa FC, Woods DJ, Fonteles MM: Implications of efavirenz for neuropsychiatry: a review. Int J Neurosci 2010;120:739-745.
28 Decloedt EH, Maartens G: Neuronal toxicity of efavirenz: a systematic review. Expert Opin Drug Saf 2013;12:841-846.
29 Gazzard B, Balkin A, Hill A: Analysis of neuropsychiatric adverse events during clinical trials of efavirenz in antiretroviral-naive patients: a systematic review. AIDS Rev 2010;12:67-75.
30 Kenedi CA, Goforth HW: A systematic review of the psychiatric side-effects of efavirenz. AIDS Behav 2011;15:1803-1818.
31 Leutscher PD, Stecher C, Storgaard M, Larsen CS: Discontinuation of efavirenz therapy in HIV patients due to neuropsychiatric adverse effects. Scand J Infect Dis 2013;45:645-651.
32 Munoz-Moreno JA, Fumaz CR, Ferrer MJ, Gonzalez-Garcia M, Molto J, Negredo E, Clotet B: Neuropsychiatric symptoms associated with efavirenz: prevalence, correlates, and management. A neurobehavioral review. AIDS Rev 2009;11:103-109.
33 Shubber Z, Calmy A, Andrieux-Meyer I, Vitoria M, Renaud-Thery F, Shaffer N, Hargreaves S, Mills EJ, Ford N: Adverse events associated with nevirapine and efavirenz-based first-line antiretroviral therapy: a systematic review and meta-analysis. AIDS 2013;27:1403-1412.
34 Sutterlin S, Vogele C, Gauggel S: Neuropsychiatric complications of efavirenz therapy: suggestions for a new research paradigm. J Neuropsychiatry Clin Neurosci 2010;22:361-369.
35 Isaacs T, Ngwanya MR, Dlamini S, Lehloenya RJ: Annular erythema and photosensitivity as manifestations of efavirenz-induced cutaneous reactions: a review of five consecutive cases. J Antimicrob Chemother 2013;68:2871-2874.
36 Leung JM, O'Brien JG, Wong HK, Winslow DL: Efavirenz-induced hypersensitivity reaction manifesting in rash and hepatitis in a Latino male. Ann Pharmacother 2008;42:425-429.
37 Mehta U, Maartens G: Is it safe to switch between efavirenz and nevirapine in the event of toxicity? Lancet Infect Dis 2007;7:733-738.
38 Perez-Molina JA, Domingo P, Martinez E, Moreno S: The role of efavirenz compared with protease inhibitors in the body fat changes associated with highly active antiretroviral therapy. J Antimicrob Chemother 2008;62:234-245.
39 Freercks RJ, Mehta U, Stead DF, Meintjes GA: Haemolytic anaemia associated with efavirenz. AIDS 2006;20:1212-1213.
40 Reekie J, Reiss P, Ledergerber B, Sedlacek D, Parczewski M, Gatell J, Katlama C, Fatkenheuer G, Lundgren JD, Mocroft A, Euro Ssg: A comparison of the long-term durability of nevirapine, efavirenz and lopinavir in routine clinical practice in Europe: a EuroSIDA study. HIV Med 2011;12:259-268.
41 Apostolova N, Gomez-Sucerquia LJ, Gortat A, Blas-Garcia A, Esplugues JV: Autophagy as a rescue mechanism in efavirenz-induced mitochondrial dysfunction: a lesson from hepatic cells. Autophagy 2011;7:1402-1404.

42 Apostolova N, Gomez-Sucerquia LJ, Gortat A, Blas-Garcia A, Esplugues JV: Compromising mitochondrial function with the antiretroviral drug efavirenz induces cell survival-promoting autophagy. Hepatology 2011;54:1009-1019.
43 Apostolova N, Gomez-Sucerquia LJ, Moran A, Alvarez A, Blas-Garcia A, Esplugues JV: Enhanced oxidative stress and increased mitochondrial mass during efavirenz-induced apoptosis in human hepatic cells. Br J Pharmacol 2010;160:2069-2084.
44 Bumpus NN: Efavirenz and 8-hydroxyefavirenz induce cell death via a JNK- and BimEL-dependent mechanism in primary human hepatocytes. Toxicol Appl Pharmacol 2011;257:227-234.
45 Polo M, Alegre F, Funes HA, Blas-Garcia A, Victor VM, Esplugues JV, Apostolova N: Mitochondrial (dys) function - a factor underlying the variability of efavirenz-induced hepatotoxicity? Br J Pharmacol 2015;172:1713-1727.
46 Lang F, Qadri SM: Mechanisms and significance of eryptosis, the suicidal death of erythrocytes. Blood Purif 2012;33:125-130.
47 Lang PA, Kaiser S, Myssina S, Wieder T, Lang F, Huber SM: Role of Ca2+-activated K+ channels in human erythrocyte apoptosis. Am J Physiol Cell Physiol 2003;285:C1553-C1560.
48 Abed M, Towhid ST, Mia S, Pakladok T, Alesutan I, Borst O, Gawaz M, Gulbins E, Lang F: Sphingomyelinase-induced adhesion of eryptotic erythrocytes to endothelial cells. Am J Physiol Cell Physiol 2012;303:C991-999.
49 Lau IP, Chen H, Wang J, Ong HC, Leung KC, Ho HP, Kong SK: In vitro effect of CTAB- and PEG-coated gold nanorods on the induction of eryptosis/erythroptosis in human erythrocytes. Nanotoxicology 2012;6:847-856.
50 Maellaro E, Leoncini S, Moretti D, Del Bello B, Tanganelli I, De Felice C, Ciccoli L: Erythrocyte caspase-3 activation and oxidative imbalance in erythrocytes and in plasma of type 2 diabetic patients. Acta Diabetol 2013;50:489-495.
51 Zelenak C, Eberhard M, Jilani K, Qadri SM, Macek B, Lang F: Protein kinase CK1alpha regulates erythrocyte survival. Cell Physiol Biochem 2012;29:171-180.
52 Bhavsar SK, Gu S, Bobbala D, Lang F: Janus kinase 3 is expressed in erythrocytes, phosphorylated upon energy depletion and involved in the regulation of suicidal erythrocyte death. Cell Physiol Biochem 2011;27:547-556.
53 Gatidis S, Zelenak C, Fajol A, Lang E, Jilani K, Michael D, Qadri SM, Lang F: p38 MAPK activation and function following osmotic shock of erythrocytes. Cell Physiol Biochem 2011;28:1279-1286.
54 Jilani K, Lang F: Carmustine-induced phosphatidylserine translocation in the erythrocyte membrane. Toxins (Basel) 2013;5:703-716.
55 Vota DM, Maltaneri RE, Wenker SD, Nesse AB, Vittori DC: Differential erythropoietin action upon cells induced to eryptosis by different agents. Cell Biochem Biophys 2013;65:145-157.
56 Zappulla D: Environmental stress, erythrocyte dysfunctions, inflammation, and the metabolic syndrome: adaptations to CO2 increases? J Cardiometab Syndr 2008;3:30-34.
57 Lupescu A, Jilani K, Zbidah M, Lang F: Patulin-induced suicidal erythrocyte death. Cell Physiol Biochem 2013;32:291-299.
58 Abed M, Zoubi KA, Theurer M, Lang F: Effect of dermaseptin on erythrocytes. Basic Clin Pharmacol Toxicol 2013;113:347-352.
59 Ahmed MS, Langer H, Abed M, Voelkl J, Lang F: The uremic toxin acrolein promotes suicidal erythrocyte death. Kidney Blood Press Res 2013;37:158-167.
60 Ghashghaeinia M, Cluitmans JC, Toulany M, Saki M, Koberle M, Lang E, Dreischer P, Biedermann T, Duszenko M, Lang F, Bosman GJ, Wieder T: Age Sensitivity of NFkappaB Abundance and Programmed Cell Death in Erythrocytes Induced by NFkappaB Inhibitors. Cell Physiol Biochem 2013;32:801-813.
61 Alzoubi K, Honisch S, Abed M, Lang F: Triggering of Suicidal Erythrocyte Death by Penta-O-galloyl-beta-d-glucose. Toxins (Basel) 2014;6:54-65.
62 Jilani K, Enkel S, Bissinger R, Almilaji A, Abed M, Lang F: Fluoxetine induced suicidal erythrocyte death. Toxins (Basel) 2013;5:1230-1243.
63 Lupescu A, Bissinger R, Jilani K, Lang F: Triggering of suicidal erythrocyte death by celecoxib. Toxins (Basel) 2013;5:1543-1554.
64 Arnold M, Lang E, Modicano P, Bissinger R, Faggio C, Abed M, Lang F: Effect of nitazoxanide on erythrocytes. Basic Clin Pharmacol Toxicol 2014;114:421-426.

65 Oswald G, Alzoubi K, Abed M, Lang F: Stimulation of suicidal erythrocyte death by ribavirin. Basic Clin Pharmacol Toxicol 2014;114:311-317.
66 Lupescu A, Bissinger R, Herrmann T, Oswald G, Jilani K, Lang F: Induction of suicidal erythrocyte death by novobiocin. Cell Physiol Biochem 2014;33:670-680.
67 Abed M, Feger M, Alzoubi K, Pakladok T, Frauenfeld L, Geiger C, Towhid ST, Lang F: Sensitization of erythrocytes to suicidal erythrocyte death following water deprivation. Kidney Blood Press Res 2013;37:567-578.
68 Alzoubi K, Calabrò S, Bissinger R, Abed M, Faggio C, Lang F: Stimulation of Suicidal Erythrocyte Death by Artesunate. Cell Physiol Biochem 2014;34:2232-2244.
69 Arnold M, Bissinger R, Lang F: Mitoxantrone-induced suicidal erythrocyte death. Cell Physiol Biochem 2014;34:1756-1767.
70 Bissinger R, Fischer S, Jilani K, Lang F: Stimulation of Erythrocyte Death by Phloretin. Cell Physiol Biochem 2014;34:2256-2265.
71 Bissinger R, Lupescu A, Zelenak C, Jilani K, Lang F: Stimulation of eryptosis by cryptotanshinone. Cell Physiol Biochem 2014;34:432-442.
72 Bissinger R, Modicano P, Frauenfeld L, Lang E, Jacobi J, Faggio C, Lang F: Estramustine-induced suicidal erythrocyte death. Cell Physiol Biochem 2013;32:1426-1436.
73 Jacobi J, Lang E, Bissinger R, Frauenfeld L, Modicano P, Faggio C, Abed M, Lang F: Stimulation of erythrocyte cell membrane scrambling by mitotane. Cell Physiol Biochem 2014;33:1516-1526.
74 Lupescu A, Bissinger R, Warsi J, Jilani K, Lang F: Stimulation of erythrocyte cell membrane scrambling by gedunin. Cell Physiol Biochem 2014;33:1838-1848.
75 Malik A, Bissinger R, Calabro S, Faggio C, Jilani K, Lang F: Aristolochic Acid Induced Suicidal Erythrocyte Death. Kidney Blood Press Res 2014;39:408-419.
76 Tesoriere L, Attanzio A, Allegra M, Cilla A, Gentile C, Livrea MA: Oxysterol mixture in hypercholesterolemia-relevant proportion causes oxidative stress-dependent eryptosis. Cell Physiol Biochem 2014;34:1075-1089.
77 Voelkl J, Alzoubi K, Mamar AK, Ahmed MS, Abed M, Lang F: Stimulation of suicidal erythrocyte death by increased extracellular phosphate concentrations. Kidney Blood Press Res 2013;38:42-51.
78 Zhang R, Xiang Y, Ran Q, Deng X, Xiao Y, Xiang L, Li Z: Involvement of calcium, reactive oxygen species, and ATP in hexavalent chromium-induced damage in red blood cells. Cell Physiol Biochem 2014;34:1780-1791.
79 Abed M, Artunc F, Alzoubi K, Honisch S, Baumann D, Foller M, Lang F: Suicidal erythrocyte death in end-stage renal disease. J Mol Med (Berl) 2014;92:871-879.
80 Polak-Jonkisz D, Purzyc L: Ca(2+) influx versus efflux during eryptosis in uremic erythrocytes. Blood Purif 2012;34:209-210; author reply 210.
81 Calderon-Salinas JV, Munoz-Reyes EG, Guerrero-Romero JF, Rodriguez-Moran M, Bracho-Riquelme RL, Carrera-Gracia MA, Quintanar-Escorza MA: Eryptosis and oxidative damage in type 2 diabetic mellitus patients with chronic kidney disease. Mol Cell Biochem 2011;357:171-179.
82 Lang PA, Beringer O, Nicolay JP, Amon O, Kempe DS, Hermle T, Attanasio P, Akel A, Schafer R, Friedrich B, Risler T, Baur M, Olbricht CJ, Zimmerhackl LB, Zipfel PF, Wieder T, Lang F: Suicidal death of erythrocytes in recurrent hemolytic uremic syndrome. J Mol Med (Berl) 2006;84:378-388.
83 Nicolay JP, Schneider J, Niemoeller OM, Artunc F, Portero-Otin M, Haik G, Jr., Thornalley PJ, Schleicher E, Wieder T, Lang F: Stimulation of suicidal erythrocyte death by methylglyoxal. Cell Physiol Biochem 2006;18:223-232.
84 Lang E, Gatidis S, Freise NF, Bock H, Kubitz R, Lauermann C, Orth HM, Klindt C, Schuier M, Keitel V, Reich M, Liu G, Schmidt S, Xu HC, Qadri SM, Herebian D, Pandyra AA, Mayatepek E, Gulbins E, Lang F, Haussinger D, Lang KS, Foller M, Lang PA: Conjugated bilirubin triggers anemia by inducing erythrocyte death. Hepatology 2015;61:275-284.
85 Qadri SM, Mahmud H, Lang E, Gu S, Bobbala D, Zelenak C, Jilani K, Siegfried A, Foller M, Lang F: Enhanced suicidal erythrocyte death in mice carrying a loss-of-function mutation of the adenomatous polyposis coli gene. J Cell Mol Med 2012;16:1085-1093.
86 Kempe DS, Akel A, Lang PA, Hermle T, Biswas R, Muresanu J, Friedrich B, Dreischer P, Wolz C, Schumacher U, Peschel A, Gotz F, Doring G, Wieder T, Gulbins E, Lang F: Suicidal erythrocyte death in sepsis. J Mol Med (Berl) 2007;85:273-281.

87 Lang KS, Roll B, Myssina S, Schittenhelm M, Scheel-Walter HG, Kanz L, Fritz J, Lang F, Huber SM, Wieder T: Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency. Cell Physiol Biochem 2002;12:365-372.
88 Lang PA, Schenck M, Nicolay JP, Becker JU, Kempe DS, Lupescu A, Koka S, Eisele K, Klarl BA, Rubben H, Schmid KW, Mann K, Hildenbrand S, Hefter H, Huber SM, Wieder T, Erhardt A, Haussinger D, Gulbins E, Lang F: Liver cell death and anemia in Wilson disease involve acid sphingomyelinase and ceramide. Nat Med 2007;13:164-170.
89 LaRocca TJ, Stivison EA, Hod EA, Spitalnik SL, Cowan PJ, Randis TM, Ratner AJ: Human-specific bacterial pore-forming toxins induce programmed necrosis in erythrocytes. MBio 2014;5:e01251-01214.
90 LaRocca TJ, Stivison EA, Mal-Sarkar T, Hooven TA, Hod EA, Spitalnik SL, Ratner AJ: CD59 signaling and membrane pores drive Syk-dependent erythrocyte necroptosis. Cell Death Dis 2015;6:e1773.
91 Kappelhoff BS, van Leth F, Robinson PA, MacGregor TR, Baraldi E, Montella F, Uip DE, Thompson MA, Russell DB, Lange JM, Beijnen JH, Huitema AD, Group NNS: Are adverse events of nevirapine and efavirenz related to plasma concentrations? Antivir Ther 2005;10:489-498.
92 Harrison HE, Bunting H, Ordway NK, Albrink WS: The Pathogenesis of the Renal Injury Produced in the Dog by Hemoglobin or Methemoglobin. J Exp Med 1947;86:339-356.
93 Kirk K: Membrane transport in the malaria-infected erythrocyte. Physiol Rev 2001;81:495-537.
94 Ayi K, Giribaldi G, Skorokhod A, Schwarzer E, Prendergast PT, Arese P: 16alpha-bromoepiandrosterone, an antimalarial analogue of the hormone dehydroepiandrosterone, enhances phagocytosis of ring stage parasitized erythrocytes: a novel mechanism for antimalarial activity. Antimicrob Agents Chemother 2002;46:3180-3184.
95 Ayi K, Turrini F, Piga A, Arese P: Enhanced phagocytosis of ring-parasitized mutant erythrocytes: a common mechanism that may explain protection against falciparum malaria in sickle trait and beta-thalassemia trait. Blood 2004;104:3364-3371.
96 Cappadoro M, Giribaldi G, O'Brien E, Turrini F, Mannu F, Ulliers D, Simula G, Luzzatto L, Arese P: Early phagocytosis of glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes parasitized by Plasmodium falciparum may explain malaria protection in G6PD deficiency. Blood 1998;92:2527-2534.
97 Koka S, Huber SM, Boini KM, Lang C, Foller M, Lang F: Lead decreases parasitemia and enhances survival of Plasmodium berghei-infected mice. Biochem Biophys Res Commun 2007;363:484-489.
98 Koka S, Lang C, Niemoeller OM, Boini KM, Nicolay JP, Huber SM, Lang F: Influence of NO synthase inhibitor L-NAME on parasitemia and survival of Plasmodium berghei infected mice. Cell Physiol Biochem 2008;21:481-488.
99 Borst O, Abed M, Alesutan I, Towhid ST, Qadri SM, Foller M, Gawaz M, Lang F: Dynamic adhesion of eryptotic erythrocytes to endothelial cells via CXCL16/SR-PSOX. Am J Physiol Cell Physiol 2012;302:C644-C651.
100 Andrews DA, Low PS: Role of red blood cells in thrombosis. Curr Opin Hematol 1999;6:76-82.
101 Chung SM, Bae ON, Lim KM, Noh JY, Lee MY, Jung YS, Chung JH: Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant microvesicle generation in human erythrocytes. Arterioscler Thromb Vasc Biol 2007;27:414-421.
102 Zwaal RF, Comfurius P, Bevers EM: Surface exposure of phosphatidylserine in pathological cells. Cell Mol Life Sci 2005;62:971-988.
103 Closse C, Dachary-Prigent J, Boisseau MR: Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. Br J Haematol 1999;107:300-302.
104 Gallagher PG, Chang SH, Rettig MP, Neely JE, Hillery CA, Smith BD, Low PS: Altered erythrocyte endothelial adherence and membrane phospholipid asymmetry in hereditary hydrocytosis. Blood 2003;101:4625-4627.
105 Pandolfi A, Di Pietro N, Sirolli V, Giardinelli A, Di Silvestre S, Amoroso L, Di Tomo P, Capani F, Consoli A, Bonomini M: Mechanisms of uremic erythrocyte-induced adhesion of human monocytes to cultured endothelial cells. J Cell Physiol 2007;213:699-709.
106 Wood BL, Gibson DF, Tait JF: Increased erythrocyte phosphatidylserine exposure in sickle cell disease: flow-cytometric measurement and clinical associations. Blood 1996;88:1873-1880.