

## Original Paper

# Stimulating Effect of Elvitegravir on Suicidal Erythrocyte Death

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Phosphatidylserine • Cell volume • Eryptosis • SB203580 • zVAD • Calcium

**Abstract**

**Background/Aims:** The antiviral drug Elvitegravir is used for the treatment of Human Immunodeficiency Virus (HIV) infections. The present study explored whether the drug is able to trigger eryptosis, the suicidal death of erythrocytes. Eryptosis is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Stimulators of eryptosis include increase of cytosolic  $Ca^{2+}$  activity ( $[Ca^{2+}]_i$ ), oxidative stress, ceramide, activated p38 kinase and activated caspases. The present study explored, whether Elvitegravir induces eryptosis and, if so, to shed light on the mechanisms involved. **Methods:** Phosphatidylserine abundance at the erythrocyte surface was estimated from annexin-V-binding, cell volume from forward scatter,  $[Ca^{2+}]_i$  from Fluo3-fluorescence, abundance of reactive oxygen species (ROS) from DCFDA dependent fluorescence, and ceramide abundance at the erythrocyte surface utilizing specific antibodies. **Results:** A 48 hours exposure of human erythrocytes to Elvitegravir ( $\geq 1.5 \mu\text{g/ml}$ ) significantly increased the percentage of annexin-V-binding cells, and significantly decreased forward scatter. Elvitegravir ( $2.5 \mu\text{g/ml}$ ) significantly increased Fluo3-fluorescence, but did not significantly modify DCFDA fluorescence or ceramide abundance. The effect of Elvitegravir on annexin-V-binding was significantly blunted by removal of extracellular  $Ca^{2+}$ , but not in the presence of p38 kinase inhibitor SB203580 ( $2 \mu\text{M}$ ) or in the presence of pancaspase inhibitor zVAD ( $10 \mu\text{M}$ ). **Conclusions:** Elvitegravir triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect in part due to entry of extracellular  $Ca^{2+}$ .

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Published by S. Karger AG, Basel**Introduction**

The viral integrase inhibitor [1] Elvitegravir is an antiretroviral drug [1-5] used for the treatment of Human Immunodeficiency Virus (HIV) infections [1, 2, 4-16].

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Several related other antiviral drugs, such as nelfinavir [17], saquinavir [18] and lopinavir [19] have been shown to trigger eryptosis, the suicidal death of erythrocytes characterized by cell shrinkage [20] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [21]. Signaling underlying stimulation of eryptosis include  $\text{Ca}^{2+}$  entry with increase of cytosolic  $\text{Ca}^{2+}$  activity ( $[\text{Ca}^{2+}]_i$ ) [21], ceramide [22], caspases [21, 23, 24], as well as several kinases including casein kinase 1 $\alpha$ , Janus-activated kinase JAK3, protein kinase C, and p38 kinase [21]. Eryptosis is inhibited by AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase, mitogen- and stress-activated kinase MSK1/2 and sorafenib/sunitinib sensitive kinases [21, 25]. Eryptosis is triggered by oxidative stress [21], energy depletion [21] and a wide variety of xenobiotics [18, 21, 26-55].

The present study explored, whether Elvitegravir stimulates eryptosis. To this end, human erythrocytes drawn from healthy volunteers were treated with Elvitegravir and phosphatidylserine surface abundance, cell volume,  $[\text{Ca}^{2+}]_i$ , abundance of reactive oxygen species (ROS) and ceramide 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 for 48 hours at 37°C *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO<sub>4</sub>, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, and 1 CaCl<sub>2</sub>. Where indicated, erythrocytes were exposed for 48 hours to Elvitegravir (MedChem Express, Princeton, USA). To test for an involvement of p38 kinase, erythrocytes were exposed for 48 hours to a combination of Elvitegravir and p38 kinase inhibitor SB203580 (Tocris bioscience, Bristol, UK). To test for an involvement of caspases, erythrocytes were exposed for 48 hours to a combination of Elvitegravir and pancaspase inhibitor zVAD (Tocris bioscience, Bristol, UK).

### *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<sub>2</sub> 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 Elvitegravir 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<sup>2+</sup>*

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<sub>2</sub> 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<sub>2</sub>. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer solution. Then, Ca<sup>2+</sup>-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<sup>2+</sup> dependent fluorescence was determined.

### *Reactive oxidant 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  $\mu$ l 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  $\mu$ g/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.

#### Statistics

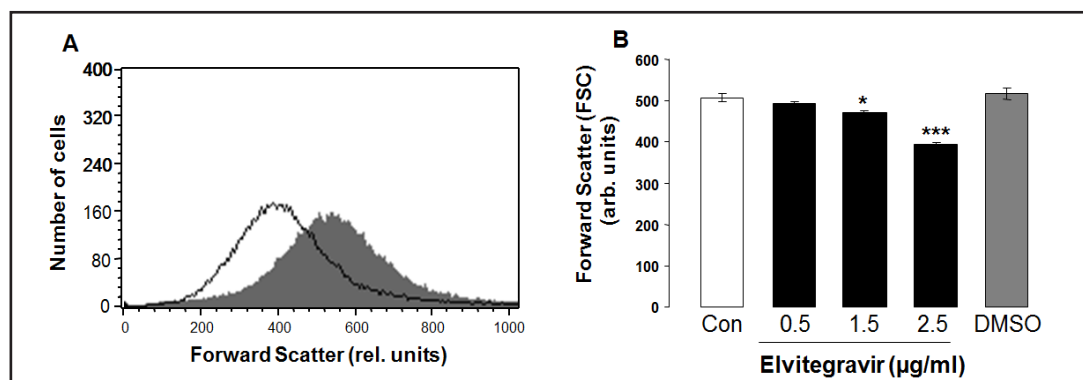
Data are expressed as arithmetic means  $\pm$  SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and *t* test as appropriate. *n* denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in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

## Results

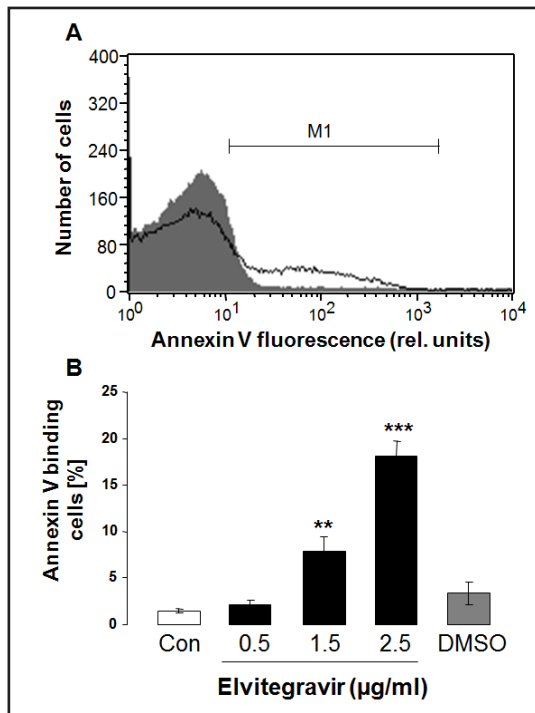
The present study tested whether Elvitegravir stimulates eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and by phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Erythrocyte volume was estimated from forward scatter, which was determined utilizing flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with Elvitegravir (0.5 – 2.5  $\mu$ g/ml). As illustrated in Fig. 1, Elvitegravir decreased erythrocyte forward scatter, an effect reaching statistical significance at 1.5  $\mu$ g/ml Elvitegravir concentration.

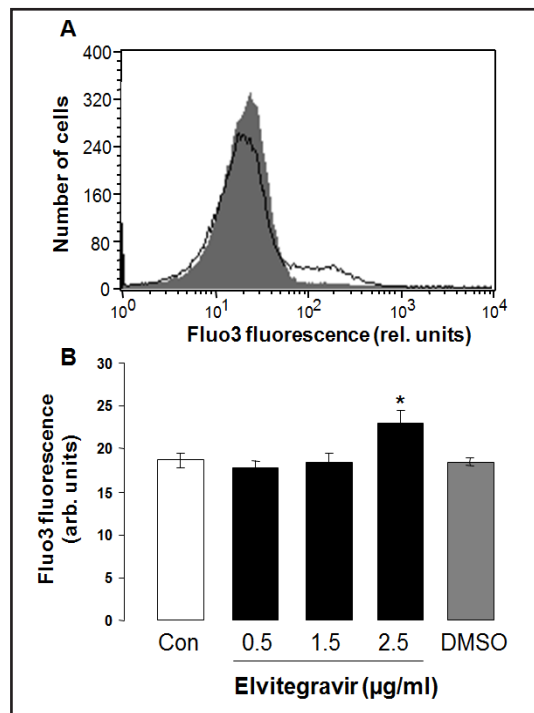
Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. Prior to measurements, the erythrocytes were again



**Fig. 1.** Effect of Elvitegravir 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 2.5  $\mu$ g/ml Elvitegravir. B. Arithmetic means  $\pm$  SEM (*n* = 10) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Elvitegravir (0.5 - 2.5  $\mu$ g/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). \* (*p* < 0.05), \*\*\* (*p* < 0.001) indicate significant difference from the absence of Elvitegravir (ANOVA).



**Fig. 2.** Effect of Elvitegravir 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 2.5 µg/ml Elvitegravir. B. Arithmetic means ± SEM (n = 10) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Elvitegravir (0.5 - 2.5 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). \*\*( $p < 0.01$ ), \*\*\*( $p < 0.001$ ) indicate significant difference from the absence of Elvitegravir (ANOVA).



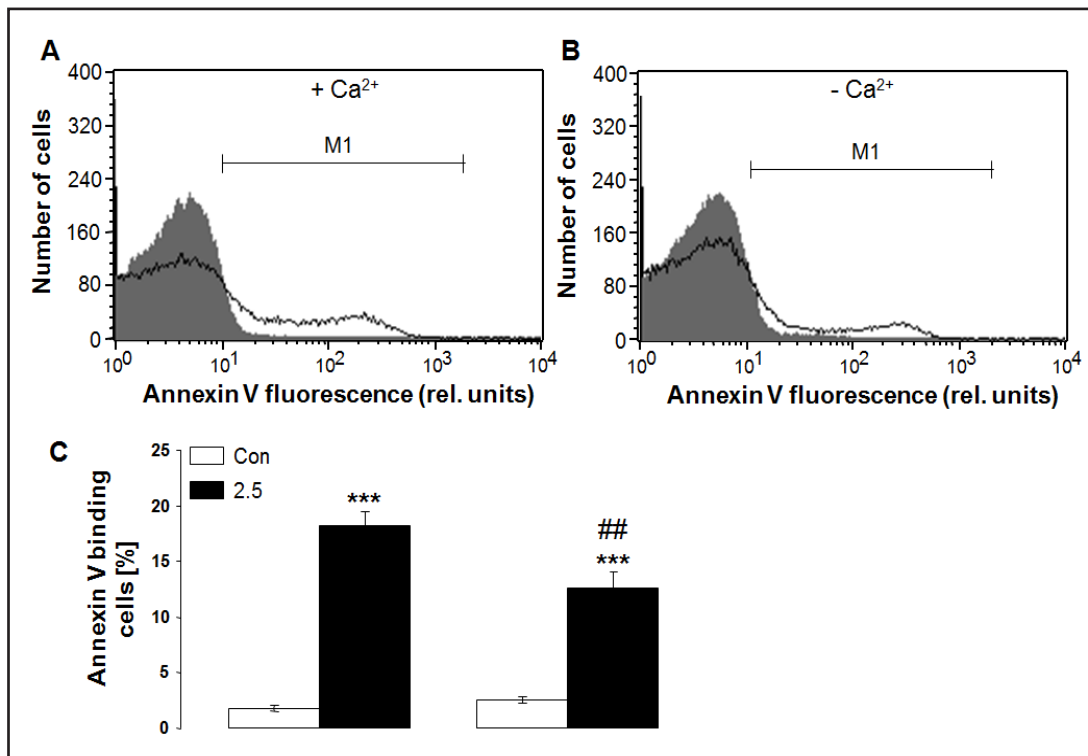
**Fig. 3.** Effect of Elvitegravir on cytosolic  $\text{Ca}^{2+}$  activity. A. Original histogram of Fluo3 fluorescence of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 2.5 µg/ml Elvitegravir. B. Arithmetic means ± SEM (n = 10) of Fluo3 fluorescence in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Elvitegravir (0.5 - 2.5 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). \*( $p < 0.05$ ) indicates significant difference from the absence of Elvitegravir (ANOVA).

incubated for 48 hours in Ringer solution without or with Elvitegravir (0.5 – 2.5 µg/ml). As illustrated in Fig. 2, a 48 hours exposure to Elvitegravir increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 1.5 µg/ml Elvitegravir.

Fluo3 fluorescence was taken as a measure of cytosolic  $\text{Ca}^{2+}$  activity ( $[\text{Ca}^{2+}]_i$ ). As illustrated in Fig. 3, a 48 hours exposure to 2.5 µg/ml Elvitegravir significantly increased the Fluo3 fluorescence, an observation pointing to increase of  $[\text{Ca}^{2+}]_i$ .

A next series of experiments explored whether the Elvitegravir-induced translocation of phosphatidylserine was sensitive to extracellular  $\text{Ca}^{2+}$ . To this end, erythrocytes were incubated for 48 hours in the absence or presence of 2.5 µg/ml Elvitegravir in the presence or nominal absence of extracellular  $\text{Ca}^{2+}$ . As shown in Fig. 4, removal of extracellular  $\text{Ca}^{2+}$  significantly blunted the effect of Elvitegravir on the percentage of annexin-V-binding erythrocytes. However, even in the absence of extracellular  $\text{Ca}^{2+}$ , Elvitegravir significantly increased the percentage of annexin-V-binding erythrocytes. Thus, Elvitegravir-induced cell shrinkage was in part, but not fully due to entry of extracellular  $\text{Ca}^{2+}$ .

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As a result, the



**Fig. 4.** Ca<sup>2+</sup> sensitivity of Elvitegravir-induced phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) Elvitegravir (2.5 μg/ml) in the presence (A) and absence (B) of extracellular Ca<sup>2+</sup>. 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) Elvitegravir (2.5 μg/ml) in the presence (left bars, +Ca<sup>2+</sup>) and absence (right bars, -Ca<sup>2+</sup>) of Ca<sup>2+</sup>. \*\*\*(*p*<0.001) indicates significant difference from the absence of Elvitegravir, ##(*p*<0.01) indicates significant difference from the presence of Ca<sup>2+</sup> (ANOVA).

DCFDA fluorescence was similar following exposure to 2.5 μg/ml Elvitegravir (17.7 ± 0.8 a.u., n = 11) and in the absence of Elvitegravir (16.9 ± 0.8 a.u., n = 11). Thus, Elvitegravir did not appreciably induce oxidative stress.

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As a result, the ceramide abundance was similar following exposure to 2.5 μg/ml Elvitegravir (16.6 ± 0.7 a.u., n = 11) and in the absence of Elvitegravir (16.7 ± 0.7 a.u., n = 11). Thus, Elvitegravir did not appreciably induce ceramide abundance.

To explore, whether the effects of Elvitegravir involved p38 kinase activity, the influence of Elvitegravir on annexin-V-binding was tested in the absence or presence of p38 kinase inhibitor SB203580 (2 μM). As a result, Elvitegravir (2.5 μg/ml) increased the percentage of phosphatidylserine exposing erythrocytes to similar values in the absence (from 2.1 ± 0.1 % to 20.8 ± 1.5 %, n = 5) and in the presence (from 2.4 ± 0.2 % to 21.6 ± 1.5 %, n = 5) of SB203580 (2 μM).

A final series of experiments addressed the putative involvement of caspases in Elvitegravir induced eryptosis. To this end, the influence of Elvitegravir on annexin-V-binding was tested in the absence or presence of pancaspase inhibitor zVAD (10 μM). As a result, Elvitegravir (2.5 μg/ml) increased the percentage of phosphatidylserine exposing erythrocytes to similar values in the absence (from 2.4 ± 0.7 % to 21.8 ± 2.1 %, n = 5) and in the presence (from 2.0 ± 0.5 % to 21.7 ± 2.5 %, n = 5) of zVAD (10 μM).



## Discussion

The present observations reveal a novel effect of Elvitegravir, i.e. the stimulation of eryptosis, the suicidal erythrocyte death. Treatment of erythrocytes from healthy individuals with Elvitegravir results in cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentrations required for this effect are in the range of peak plasma concentrations determined in Elvitegravir treated patients [56]. A single dose of 150 mg of Elvitegravir has been shown to be followed by an increase of the plasma Elvitegravir concentration to 2.3 µg/ml and a subsequent decline of the plasma Elvitegravir concentration to 0.5 µg/ml [56]. At least in theory, the sensitivity to Elvitegravir induced eryptosis could be enhanced in clinical conditions with accelerated eryptosis, such as dehydration [57], hyperphosphatemia [58], chronic kidney disease (CKD) [59-62], hemolytic-uremic syndrome [63], diabetes [64], hepatic failure [65], malignancy [21], sepsis [66], sickle-cell disease [21], beta-thalassemia [21], Hb-C and G6PD-deficiency [21], as well as Wilsons disease [67].

The effect of Elvitegravir on cell membrane scrambling was blunted by removal of Ca<sup>2+</sup> from the extracellular space, indicating that the effect was partially dependent on entry of extracellular Ca<sup>2+</sup>. The effect of Elvitegravir on cell shrinkage was presumably as well partially due to Ca<sup>2+</sup> entry from the extracellular space leading to increase of [Ca<sup>2+</sup>]<sub>i</sub> with subsequent activation of Ca<sup>2+</sup> sensitive K<sup>+</sup> channels, K<sup>+</sup> exit, cell membrane hyperpolarization, Cl<sup>-</sup> exit and thus cellular loss of KCl with water [20].

However, Elvitegravir triggered cell membrane scrambling even in the absence of extracellular Ca<sup>2+</sup>, indicating that Elvitegravir triggered cell membrane scrambling in part by mechanisms other than Ca<sup>2+</sup> entry. Additional mechanisms triggering eryptosis include oxidative stress, ceramide, activation of p38 kinase or stimulation of caspases [21]. However, Elvitegravir triggered cell membrane scrambling and cell shrinkage without enhancing the ceramide abundance and without inducing oxidative stress. Moreover, the effect of Elvitegravir was insensitive to inhibition of p38 kinase with SB203580 and to inhibition of caspases with zVAD. Thus, the additional mechanism involved in the triggering of eryptosis other than Ca<sup>2+</sup> entry remained elusive.

Eryptosis allows the clearance of defective erythrocytes from circulating blood prior to hemolysis [21]. Avoidance of hemolysis is important as hemolysis leads to release of hemoglobin, which passes the renal glomerular filter, precipitates in the acidic lumen of renal tubules, occludes nephrons and thus may lead to renal failure [68]. Eryptosis further counteracts development of parasitemia in malaria, as it is triggered by infection of host erythrocytes and accomplishes elimination of infected erythrocytes.

The clearance of phosphatidylserine exposing erythrocytes from circulating blood may lead to anemia if the loss of erythrocytes outcasts the formation of new erythrocytes by erythropoiesis [21]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [69], stimulate blood clotting and thus foster thrombosis [70-72]. Accordingly, stimulation of eryptosis may lead to impairment of microcirculation [22, 70, 73-76].

In conclusion, Elvitegravir triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect in part due to Ca<sup>2+</sup> entry.

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## Disclosure Statement

The authors of this manuscript state that they have no conflicts of interest to declare.

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