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Original Paper

Enhanced Eryptosis Following Exposure to Dolutegravir

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Key Words
Phosphatidylserine • Cell volume • Eryptosis • Ceramide • Calcium • Oxidative stress

Abstract
Background/Aims: The viral integrase enzyme inhibitor dolutegravir is utilized for the treatment of immunodeficiency virus (HIV) infection. Knowledge on cytotoxicity of dolutegravir is limited. The present study thus explored, whether dolutegravir is able to trigger suicidal erythrocyte death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Cellular mechanisms involved in the triggering of eryptosis include increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), oxidative stress, ceramide, and activation of protein kinase C, p38 kinase, casein kinase, and caspases. The present study explored, whether Dolutegravir induces eryptosis and, if so, to gain insight into cellular mechanisms involved. ***Methods:*** Utilizing flow cytometry, 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 specific antibodies. Hemolysis was quantified from haemoglobin concentration in the supernatant. ***Results:*** A 48 hours exposure of human erythrocytes to dolutegravir significantly increased the percentage of annexin-V-binding cells (≥ 4.8 μM), significantly increased hemolysis (19.1 μM), but did not significantly modify forward scatter. Dolutegravir significantly increased Fluo3-fluorescence (≥ 4.8 μM), DCFDA fluorescence (19.1 μM) and ceramide abundance (19.1 μM). The effect of dolutegravir on annexin-V-binding was significantly blunted by removal of extracellular Ca^{2+}, but was not significantly modified by protein kinase C inhibitor staurosporine (1 μM), p38 kinase inhibitor SB203580 (2 μM), casein kinase inhibitor D4476 (10 μM) or pancaspase inhibitor zVAD (10 μM). ***Conclusions:*** Dolutegravir triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect at least in part due to Ca^{2+} entry, ceramide formation and oxidative stress.

Introduction

Dolutegravir, a viral integrase enzyme inhibitor [1-24], is effective in the treatment of human immunodeficiency virus (HIV) infection [1-14, 17, 19-27]. Side effects include

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inhibition of the organic cation transporter 2 with decrease of creatinine clearance, diarrhea, headache, nausea, and insomnia [4, 17, 19, 20, 28].

Little is known about cytotoxicity of dolutegravir. A wide variety of xenobiotics [29-68] including antiviral drugs [69, 70] trigger eryptosis [56], the suicidal death of erythrocytes characterized by cell shrinkage [71] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [56]. Induction of eryptosis has thus proven a sensitive measure of cytotoxicity [56]. Cellular mechanisms leading to eryptosis include increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) [56], ceramide [72], oxidative stress [56, 73, 74], energy depletion [56], caspases [56, 75, 76], casein kinase 1α, Janus-activated kinase JAK3, protein kinase C, and p38 kinase [56]. Cellular mechanisms counteracting eryptosis include AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase [56], cyclin-dependent kinase CDK4 [77], mitogen- and stress-activated kinase MSK1/2 [78], and sorafenib/sunitinib sensitive kinases [56].

The present study tested, whether dolutegravir stimulates eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to dolutegravir and phosphatidylserine surface abundance, cell volume, $[Ca^{2+}]_i$, ROS formation, and ceramide abundance determined by flow cytometry.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Li-Heparin-anticoagulated blood samples were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003 V). The blood was centrifuged at 120 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. Where indicated, erythrocytes were exposed for 48 hours to dolutegravir (MedChem Express, Princeton, USA). To test for an involvement of kinases, erythrocytes were exposed for 48 hours to a combination of dolutegravir and p38 kinase inhibitor SB203580 (Tocris bioscience, Bristol, UK), casein kinase inhibitor D4476 (Tocris bioscience, Bristol, UK) and protein kinase C inhibitor staurosporine (Enzo Life Sciences, Lörrach, Germany). The putative involvement of caspases was tested by the use of the pancaspase inhibitor zVAD (Enzo Life Sciences, Lörrach, Germany).

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 µl cell suspension 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 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 dolutegravir 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".

Hemolysis

Following incubation, the erythrocyte suspension was centrifuged for 3 min at 1600 rpm, 4°C, and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatants was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

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. 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. Afterwards, the geomean of the Ca^{2+} dependent fluorescence was determined.

Reactive oxygen species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 µl suspension of erythrocytes was washed in Ringer solution and 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 washed two times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 µl 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, a 100 µl suspension of erythrocytes was 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. The cell suspension was subsequently 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 analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. As a control, secondary antibody alone was used. Finally, the geomean of the ceramide-dependent fluorescence was determined.

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, whether dolutegravir stimulates eryptosis, the suicidal erythrocyte death. A 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, as determined by flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with dolutegravir (4.77-19.08 µM). As shown in Fig. 1, a 48 hours exposure to dolutegravir increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 4.77 µM dolutegravir.

Hemoglobin concentration in the supernatant was determined in order to quantify hemolysis. As shown in Fig. 2, a 48 hours exposure to dolutegravir increased the percentage of hemolytic erythrocytes, an effect reaching statistical significance at 19.08 µM dolutegravir.

A second hallmark of eryptosis is cell shrinkage. Erythrocyte volume was thus estimated from forward scatter which was determined utilizing flow cytometry. The measurements were again performed after incubation of the erythrocytes for 48 hours in Ringer solution without or with dolutegravir (4.77-19.08 µM). As a result, after incubation for 48 hours, the forward scatter was similar in Ringer (515.8 ± 6.2, n = 19), in DMSO (531.9 ± 11.9, n = 19), in 4.77 µM dolutegravir (528.0 ± 10.9, n = 19), in 9.54 µM dolutegravir (519.2 ± 9.5, n = 19) and in 19.08 µM dolutegravir (515.6 ± 8.1, n = 19). Thus, dolutegravir did not significantly modify erythrocyte volume.

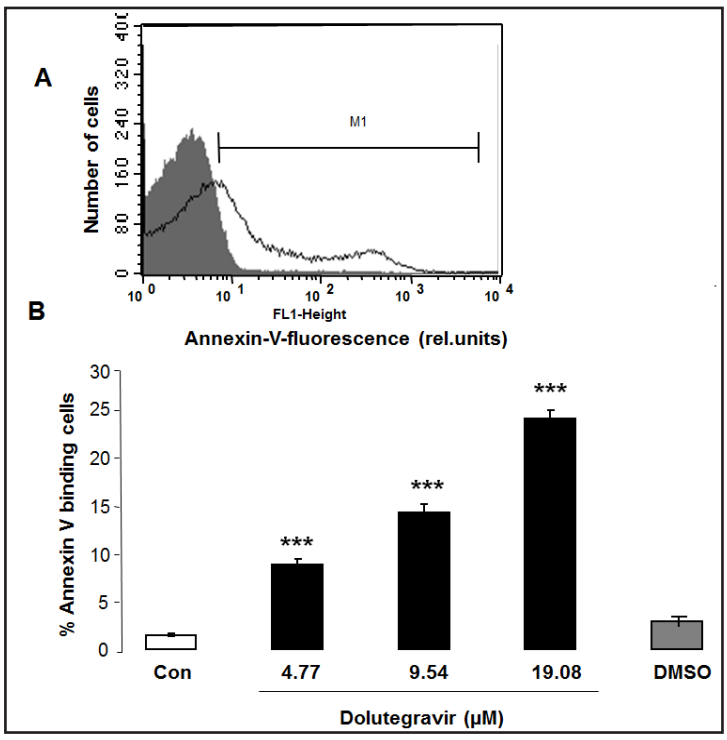

Fig. 1. Effect of dolutegravir on phosphatidylserine exposure. A. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 19.08 μM dolutegravir. B. Arithmetic means ± SEM (n = 19) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) dolutegravir (4.77-19.08 μM). For comparison, the effect of the solvent DMSO is shown (grey bar). ***(p<0.001) indicates significant difference from the absence of dolutegravir (ANOVA).

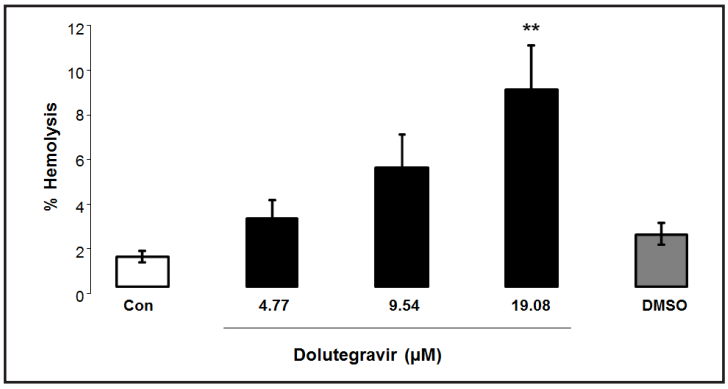

Fig. 2. Effect of Dolutegravir on hemolysis. Arithmetic means ± SEM (n = 5) of the percentage of hemolytic erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) dolutegravir (4.77-19.08 μM). For comparison, the effect of the solvent DMSO is shown (grey bar). **(p<0.01) indicates significant difference from the absence of dolutegravir (ANOVA).

Fluo3 fluorescence was taken as a measure of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). As illustrated in Fig. 3, following 48 hours incubation, the Fluo3 fluorescence was larger in the presence than in the absence of dolutegravir, a difference reaching statistical significance at 4.77 μM dolutegravir concentration.

A next series of experiments explored, whether the dolutegravir-induced cell shrinkage and translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular Ca^{2+}. To this end, erythrocytes were incubated for 48 hours in the absence or presence of 19.08 μM dolutegravir 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 dolutegravir on the percentage of annexin-V-binding erythrocytes. However, even in the absence of extracellular Ca^{2+}, dolutegravir significantly increased the percentage of annexin-V-binding erythrocytes. Thus, dolutegravir-induced cell membrane scrambling was in large part but not fully dependent on entry of extracellular Ca^{2+}.

In order to explore the involvement of kinases in the stimulation of eryptosis by dolutegravir, experiments were repeated in the presence and absence of the respective kinase inhibitors. As a result, the increase of annexin-V-binding erythrocytes following treatment with dolutegravir

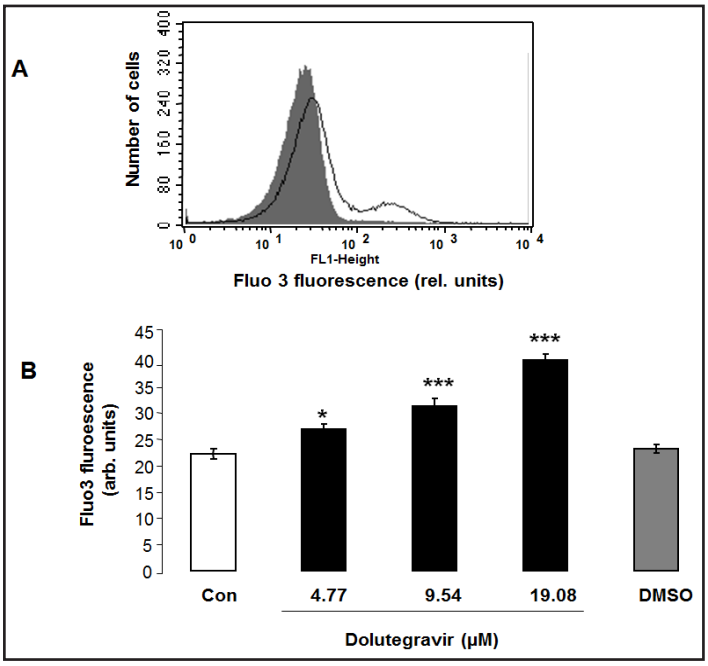

Fig. 3. Effect of Dolutegravir on cytosolic Ca^{2+} activity. A. Original histograms of Fluo3-fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 19.08 μM dolutegravir. B. Arithmetic means ± SEM (n = 19) of erythrocyte Fluo3 fluorescence following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) dolutegravir (4.77-19.08 μM). 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 dolutegravir (ANOVA).

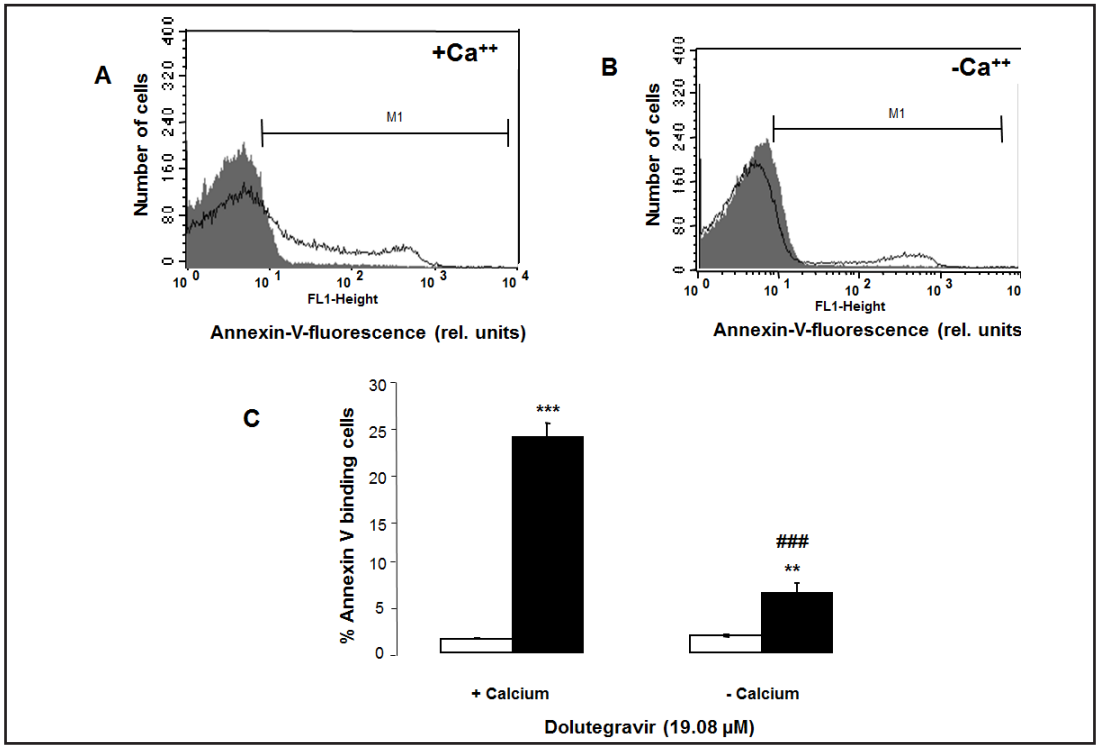

Fig. 4. Ca^{2+} sensitivity of dolutegravir-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) dolutegravir (19.08 μM) in the presence (A) and absence (B) of extracellular Ca^{2+}. C. Arithmetic means ± SEM (n = 14) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) dolutegravir (19.08 μM) 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 dolutegravir, ###(p<0.001) indicates significant difference from the presence of Ca^{2+} (ANOVA).

(19.08 μM) was similar in the absence (from 1.9 ± 0.4 % [n = 5] to 23.0 ± 2.7 % [n = 5]) and in the presence (from 1.9 ± 0.3 % [n = 5] to 21.8 ± 1.7 % [n = 5]) of p38 kinase inhibitor SB 203580

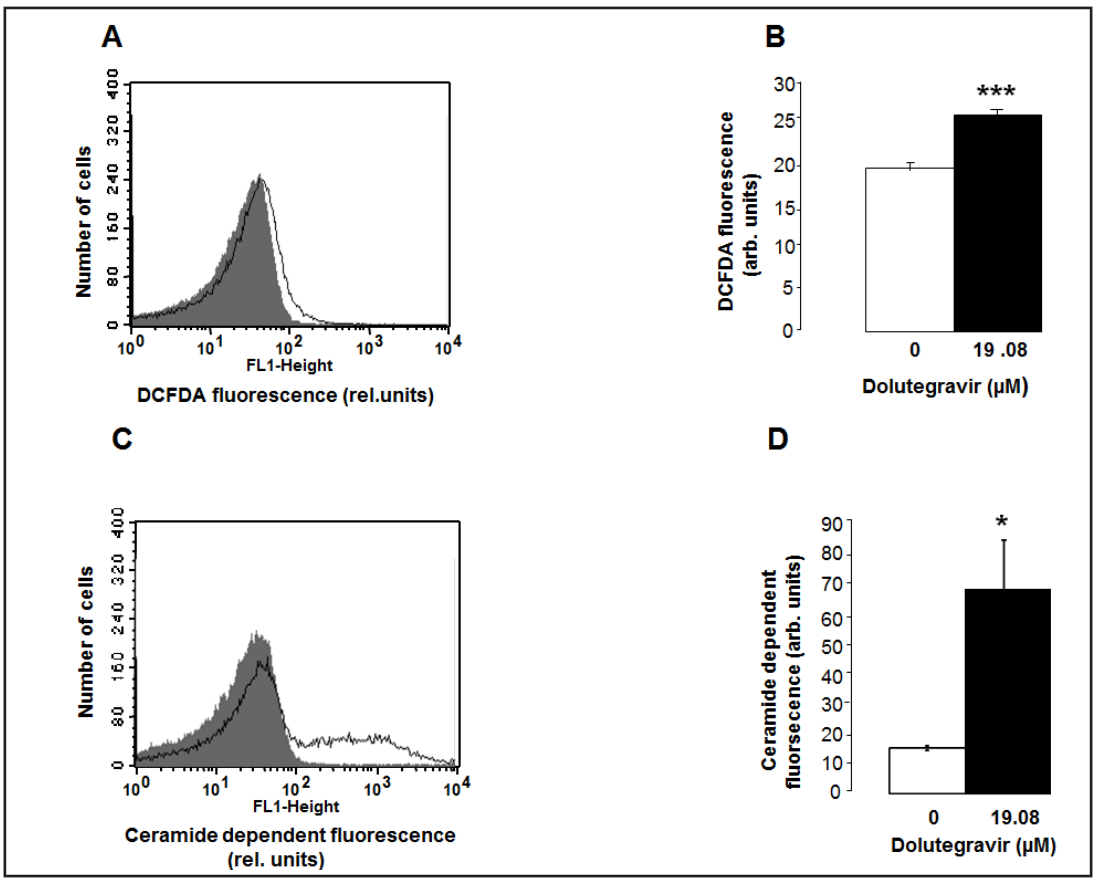

Fig. 5. Effect of dolutegravir on erythrocyte ROS formation and ceramide abundance at the erythrocyte surface. A. Original histograms of DCFDA fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of dolutegravir (19.08 μM). B. Arithmetic means ± SEM (n = 10) of the DCFDA fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) dolutegravir (19.08 μM). ***($p<0.001$) indicates significant difference from the absence of dolutegravir (unpaired *t* test). C. Original histograms of ceramide abundance in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 19.08 μM dolutegravir. D. Arithmetic means ± SEM (n = 6) of the ceramide abundance (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) presence of 19.08 μM dolutegravir. *($p<0.05$) indicates significant difference from the absence of dolutegravir (unpaired *t* test).

(2 μM). The increase of annexin-V-binding erythrocytes following treatment with dolutegravir (19.08 μM) was further similar in the absence (from 1.5 ± 0.2 % [n = 15] to 20.6 ± 1.8 % [n = 15]) and in the presence (from 1.4 ± 0.1 % [n = 15] to 20.7 ± 1.8 % [n = 15]) of casein kinase inhibitor D4476 (10 μM). Moreover, the increase of annexin-V-binding erythrocytes following treatment with dolutegravir (19.08 μM) was similar in the absence (from 2.1 ± 0.5 % [n = 5] to 23.1 ± 2.1 % [n = 5]) and in the presence (from 6.0 ± 0.9 % [n = 5] to 25.2 ± 1.7 % [n = 5]) of protein kinase C inhibitor staurosporine (1 μM).

The putative involvement of caspases was tested utilizing pancaspase inhibitor zVAD. As a result, the increase of annexin-V-binding erythrocytes following treatment with dolutegravir (19.08 μM) was similar in the absence (from 1.5 ± 0.2 % [n = 14] to 23.2 ± 1.5 % [n = 14]) and in the presence (from 1.8 ± 0.2 % [n = 14] to 20.9 ± 1.7 % [n = 14]) of zVAD (10 μM).

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). As illustrated in Fig. 5A,B, the DCFDA fluorescence was significantly higher following exposure to dolutegravir (19.08 μM) than in the absence of dolutegravir.

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As illustrated in Fig. 5C,D, the ceramide abundance was significantly higher following exposure to dolutegravir (19.08 μM) than in the absence of dolutegravir.

Discussion

The present observations reveal a toxic effect of dolutegravir on erythrocytes. Exposure of erythrocytes drawn from healthy individuals to dolutegravir is followed by cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentrations required for the effect are well in the range of reported plasma concentrations encountered following dolutegravir administration in man [28].

The effect of dolutegravir on cell membrane scrambling was paralleled by increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), was in large part blunted by removal of extracellular Ca^{2+} and was thus partially dependent on Ca^{2+} entry from the extracellular space. Nevertheless, even in the absence of extracellular Ca^{2+}, dolutegravir stimulated cell membrane scrambling indicating that a small part of cell membrane scrambling did not require an increase of $[Ca^{2+}]_i$. Thus, dolutegravir apparently stimulated Ca^{2+} entry but by the same token sensitized the cell to the scrambling effect of Ca^{2+}. Sensitization of erythrocytes for the scrambling effect of Ca^{2+} is observed following exposure to ceramide [56]. The triggering of cell membrane scrambling by dolutegravir was indeed paralleled by increase of ceramide abundance. Moreover, dolutegravir triggered eryptosis was paralleled by oxidative stress, a well known stimulator of eryptosis [56]. The effect of dolutegravir did not require the activity of p38 kinase, casein kinase or staurosporine sensitive kinases such as protein kinase C. The dolutegravir induced cell membrane scrambling was further insensitive to the presence of pancaspase inhibitor zVAD. Collectively, Ca^{2+} entry, ceramide and oxidative stress explain most, if not all the effect of dolutegravir on erythrocyte cell membrane scrambling.

Despite the increase of $[Ca^{2+}]_i$ dolutegravir did not trigger cell shrinkage. An increase of $[Ca^{2+}]_i$ was expected to activate Ca^{2+} sensitive K^+ channels thus leading to K^+ exit, cell membrane hyperpolarization, Cl^- exit and cellular loss of KCl with water. The mechanism preventing the cell shrinkage despite the increase of $[Ca^{2+}]_i$ remained elusive. Potential mechanisms include interference with Na^+/K^+ ATPase activity with decrease of cellular K^+ and thus Ca^{2+} induced hyperpolarization.

Dolutegravir treatment is further followed by hemolysis with 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 [79]. As phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood, eryptosis may prevent hemolysis *in vivo* by removing the defective erythrocytes prior to hemolysis. Eryptosis may further serve to eliminate erythrocytes infected with the malaria pathogen *Plasmodium.* Excessive eryptosis may, however, lead to anemia as soon as the loss of erythrocytes surpasses the formation of new erythrocytes by erythropoiesis [56]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [80], stimulate blood clotting and trigger thrombosis [81-83], thus impairing microcirculation [72, 81, 84-87]

The sensitivity to dolutegravir may be enhanced in clinical conditions with accelerated eryptosis, such as dehydration [88], hyperphosphatemia [89], chronic kidney disease (CKD) [90-93], hemolytic-uremic syndrome [94], diabetes [95], hepatic failure [96], malignancy [56], sepsis [97], sickle-cell disease [56], beta-thalassemia [56], Hb-C and G6PD-deficiency [56], as well as Wilsons disease [98]. Patients with those conditions may, at least in theory, be particularly sensitive to dolutegravir-induced triggering of anemia and impairment of microcirculation.

In conclusion, dolutegravir triggers erythrocyte cell membrane scrambling, an effect apparently involving Ca^{2+} entry, oxidative stress and ceramide.

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Disclosure Statement

The authors state that they have no conflict of interest.

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