Original Paper

Saquinavir Induced Suicidal Death of Human Erythrocytes

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Key Words

Phosphatidylserine • Cell volume • Eryptosis • Oxidative stress • Calcium

Abstract

Background/Aims: The antiretroviral protease inhibitor saquinavir is used for the treatment of HIV infections. Effects of saquinavir include induction of apoptosis, the suicidal death of nucleated cells. Saquinavir treatment may further lead to anemia. In theory, anemia could result from accelerated erythrocyte loss by enhanced suicidal erythrocyte death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Stimulators of eryptosis include Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), oxidative stress with increase of reactive oxygen species (ROS) and ceramide. The present study explored, whether and how saquinavir induces eryptosis. **Methods:** To this end, flow cytometry was employed to estimate erythrocyte volume from forward scatter, phosphatidylserine exposure at the cell surface from annexin-V-binding, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, ROS abundance from DCFDA fluorescence and ceramide abundance utilizing specific antibodies. **Results:** A 48 hours exposure of human erythrocytes to saquinavir significantly decreased forward scatter ($\geq 5 \mu\text{g/ml}$), significantly increased the percentage of annexin-V-binding cells ($\geq 10 \mu\text{g/ml}$), significantly increased Fluo3-fluorescence ($15 \mu\text{g/ml}$), significantly increased DCFDA fluorescence ($15 \mu\text{g/ml}$), but did not significantly modify ceramide abundance. The effect of saquinavir on annexin-V-binding was significantly blunted, but not abolished by removal of extracellular Ca^{2+} . **Conclusions:** Saquinavir triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect in part due to stimulation of ROS formation and Ca^{2+} entry.

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Introduction

Saquinavir, an antiretroviral protease inhibitor [1, 2], is utilized for the treatment of HIV infections [2-21]. Saquinavir and its nitric oxide-derivative have been shown to trigger or foster apoptosis [22-30], an effect paralleled by inhibition of AKT-phosphorylation [22], downregulation of NF- κ B [24, 28, 29], proteotoxic stress [22], endoplasmic reticulum stress [25], autophagy [25] and mitochondrial depolarization [31].

Side effects of saquinavir include anemia [32]. In theory, anemia could result from stimulation of eryptosis [33], the suicidal death of erythrocytes characterized by cell shrinkage [34] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [33]. Signaling stimulating eryptosis includes opening of Ca^{2+} permeable unselective cation channels with subsequent Ca^{2+} entry and increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [33], oxidative stress [33], ceramide [33], energy depletion [33], caspases, casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C and p38 kinase [33]. Signaling inhibiting eryptosis includes AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase, and sorafenib/sunitinib sensitive kinases [33]. Eryptosis is stimulated by a wide variety of xenobiotics [33, 35-59].

The present study tested a putative effect of saquinavir on eryptosis. To this end, human erythrocytes from healthy volunteers were treated with saquinavir and phosphatidylserine surface abundance, cell volume as well as $[\text{Ca}^{2+}]_i$ and ROS formation 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 to saquinavir (Sigma Aldrich, Hamburg, Germany) 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 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 saquinavir 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 then 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 and washed once in Ringer solution containing 5 mM CaCl_2 . The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer. Then, 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.

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, 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 at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

Ceramide abundance

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 then analyzed 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, whether exposure of human erythrocytes to saquinavir is followed by stimulation of suicidal erythrocyte death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface.

In order to estimate erythrocyte cell volume, forward scatter was determined utilizing flow cytometry. Measurements were made following a 48 hours incubation in Ringer solution without or with saquinavir (2.5 – 15 µg/ml). As illustrated in Fig. 1, saquinavir decreased erythrocyte forward scatter, an effect reaching statistical significance at 5 µg/ml saquinavir concentration.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, which was determined by flow cytometry. Again, the erythrocytes were analysed

Fig. 1. Effect of saquinavir 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 15 µg/ml saquinavir. B. Arithmetic means ± SEM (*n* = 18) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of saquinavir (2.5 - 15 µg/ml). For comparison, presence of the solvent DMSO (grey bar). *(*P* < 0.05) and ***(*P* < 0.001) indicate significant difference from the absence of saquinavir (ANOVA).

Fig. 2. Effect of saquinavir 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 15 µg/ml saquinavir. B. Arithmetic means $\pm$ SEM ($n = 18$) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with presence of saquinavir (2.5 - 15 µg/ml) (black bars) or solvent DMSO (grey bar). ***($P < 0.001$) indicates significant difference from the absence of saquinavir (ANOVA).

Fig. 3. Effect of saquinavir 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 15 µg/ml saquinavir. B. Arithmetic means $\pm$ SEM ($n = 15$) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with presence of saquinavir (2.5 - 15 µg/ml) (black bars) or solvent DMSO (grey bar). ***($P < 0.001$) indicates significant difference from the absence of saquinavir (ANOVA).

following incubation for 48 hours in Ringer solution without or with saquinavir (2.5 - 15 µg/ml). As shown in Fig. 2, a 48 hours exposure to saquinavir increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 10 µg/ml saquinavir.

Cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) was estimated from Fluo3 fluorescence. As illustrated in Fig. 3, a 48 hours exposure to saquinavir increased the Fluo3 fluorescence, an effect reaching statistical significance at 15 µg/ml saquinavir. In order to test whether the saquinavir-induced translocation of phosphatidylserine required entry of extracellular Ca^{2+} , erythrocytes were incubated for 48 hours in the absence or presence of 15 µg/ml saquinavir in the presence or nominal absence of extracellular Ca^{2+} . As shown in Fig. 4, removal of extracellular Ca^{2+} significantly blunted the effect of saquinavir on annexin-V-binding. However, saquinavir significantly increased the percentage of annexin-V-binding erythrocytes even in the absence of extracellular Ca^{2+} . Thus, saquinavir-induced cell membrane scrambling was in large part but not exclusively due to entry of extracellular Ca^{2+} .

Stimulators of Ca^{2+} entry and eryptosis include oxidative stress. The abundance of reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As shown in Fig. 5, a 48 hours exposure to saquinavir (15 µg/ml) significantly increased the DCFDA fluorescence, an observation pointing to induction of oxidative stress.

Fig. 4. Ca²⁺ sensitivity of saquinavir-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) presence of 15 µg/ml saquinavir in the presence (A) and absence (B) of extracellular Ca²⁺. C. Arithmetic means ± SEM (n = 5) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) presence of 15 µg/ml saquinavir in the presence (left bars, +Ca²⁺) and absence (right bars, -Ca²⁺) of Ca²⁺. ***(*P*<0.001) indicates significant difference from the absence of saquinavir, ###(*P*<0.001) indicates significant difference from the presence of Ca²⁺ (ANOVA).

Fig. 5. Effect of saquinavir on erythrocyte ROS formation. A. Original histogram of DCFDA fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 15 µg/ml saquinavir. B. Arithmetic means ± SEM (n = 11) of the DCFDA fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) and with (black bar) presence of 15 µg/ml saquinavir. ***(*P*<0.001) indicates significant difference from the absence of saquinavir (paired *t* test).

As eryptosis is further stimulated by ceramide, the ceramide abundance was quantified utilizing specific antibodies. As a result, following a 48 hours incubation, the ceramide abundance was similar in the absence (15.2 ± 0.3 , n = 11) and in the presence of saquinavir (14.3 ± 0.4 , n = 11). Thus, saquinavir did not significantly modify the ceramide abundance.

Discussion

The present observations reveal a novel effect of saquinavir, i.e. the triggering of suicidal erythrocyte death or eryptosis. Exposure of human erythrocytes to saquinavir is followed by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface.

The effect of saquinavir on eryptosis is apparently in large part due to Ca^{2+} entry from the extracellular space with increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). An increase of $[\text{Ca}^{2+}]_i$ is known to stimulate cell membrane scrambling by activating an illdefined scramblase [33]. An increase of $[\text{Ca}^{2+}]_i$ is further followed by erythrocyte shrinkage due to activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [34].

The Ca^{2+} entry from the extracellular space presumably results from activation of Ca^{2+} permeable cation channels, which are known to be activated by oxidative stress [33]. According to DCFDA fluorescence, saquinavir treatment does increase the abundance of reactive oxygen species. Saquinavir induced eryptosis is presumably not the result of ceramide formation.

As phosphatidylserine exposing erythrocytes are engulfed by phagocytosing cells, stimulation of eryptosis leads to the rapid clearance of the affected erythrocytes [33]. The removal of eryptotic erythrocytes precedes and may thus prevent hemolysis of defective erythrocytes with release of hemoglobin, which may undergo filtration in renal glomeruli, precipitate in the acidic lumen of renal tubules, occlude nephrons and thus compromise renal function [60]. In malaria, eryptosis further initiates the clearance of *Plasmodium* infected erythrocytes. The pathogen activates Ca^{2+} -permeable erythrocyte cation channels by imposing oxidative stress on the infected host cell [33, 61]. The clearance of eryptotic infected erythrocytes counteracts the development of parasitemia and thus favourably influences the clinical course of malaria. Presumably due to enhanced susceptibility to eryptosis, sickle-cell trait, beta-thalassemia-trait, Hb-C and G6PD-deficiency protect against a severe course of malaria [33, 62-64]. The enhanced eryptosis counteracts the increase of parasitemia and thus favourably influences the clinical course of malaria further in iron deficiency [65] and during treatment with lead [65], chlorpromazine [66] or NO synthase inhibitors [66]. Possibly, saquinavir similarly enhances the susceptibility of *Plasmodium* infected erythrocytes to eryptosis.

In any case, the observed stimulation of eryptosis presumably contributes to the saquinavir induced anemia [32], as the concentrations required to stimulate eryptosis are well in the range of concentrations encountered *in vivo* [22, 67]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [68], stimulate blood clotting and trigger thrombosis [69-71], thus impairing microcirculation [69, 72-76]. Saquinavir sensitivity may be enhanced in clinical conditions with stimulated eryptosis, such as dehydration [48], hyperphosphatemia [58], chronic kidney disease (CKD) [40, 77-79], hemolytic-uremic syndrome [80], diabetes [81], hepatic failure [82], malignancy [33], sepsis [83], sickle-cell disease [33], beta-thalassemia [33], Hb-C and G6PD-deficiency [33], as well as Wilsons disease [84].

In conclusion, saquinavir triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect paralleled by and in part due to induction of oxidative stress and increase of cytosolic Ca^{2+} activity.

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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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