Original Paper

Enhanced Eryptosis Following Exposure to Lopinavir

Rosi Bissinger^a Sabrina Waibel^a Ghada Bouguerra^{a,b} Abdulla Al Mamun Bhuyan^a
Salem Abbès^b Florian Lang^a

^aDepartment of Physiology, University of Tübingen, Tuebingen, Germany; ^bLaboratoire d'Hématologie Moléculaire et Cellulaire, Institut Pasteur de Tunis, Tunis, Tunisie

Key Words

Phosphatidylserine • Cell volume • Eryptosis • Oxidative stress • Calcium

Abstract

Background/Aims: The protease inhibitor lopinavir, used for the treatment of HIV infections, triggers suicidal death or apoptosis of nucleated cells. Side effects of lopinavir include anemia, which could in theory result from stimulation of suicidal erythrocyte death or eryptosis, characterized by cell shrinkage and by phospholipid scrambling of the cell membrane leading to phosphatidylserine translocation to the erythrocyte surface. Stimulators of eryptosis include oxidative stress, increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), and ceramide. The present study explored, whether lopinavir induces eryptosis. **Methods:** Flow cytometry was employed to estimate phosphatidylserine exposure at the cell surface from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, reactive oxygen species (ROS) abundance from 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence, reduced glutathione (GSH) from mercury orange fluorescence and ceramide abundance utilizing labelled specific antibodies. Hemolysis was estimated from haemoglobin concentration of the supernatant. **Results:** A 48 hours exposure of human erythrocytes to lopinavir significantly increased the percentage of annexin-V-binding cells ($\geq 10 \mu\text{g/ml}$), significantly decreased forward scatter ($\geq 15 \mu\text{g/ml}$), significantly increased hemolysis ($\geq 15 \mu\text{g/ml}$), significantly increased Fluo3-fluorescence ($20 \mu\text{g/ml}$), and significantly increased DCFDA fluorescence ($20 \mu\text{g/ml}$) but did not significantly modify ceramide abundance. The effect of lopinavir on annexin-V-binding was significantly blunted, but not abolished by removal of extracellular Ca^{2+} . **Conclusion:** Lopinavir treatment of erythrocytes from healthy volunteers is followed by cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect in part due to stimulation of ROS formation and Ca^{2+} entry.

© 2015 The Author(s)
Published by S. Karger AG, Basel

R. Bissinger and S. Waibel contributed equally and thus share first authorship

Prof. Dr. Florian Lang

Physiologisches Institut der Universität Tübingen, Gmelinstr. 5, D-72076 Tübingen (Germany)
Tel. +49 7071 29 72194, Fax +49 7071 29 5618, E-Mail florian.lang@uni-tuebingen.de

Introduction

The protease inhibitor lopinavir is used for the treatment of HIV infections [1-13]. Side effects of lopinavir treatment include anemia [14]. Lopinavir may induce proteotoxic stress [15, 16], trigger oxidative stress [16], and suppress NF- κ B activity [17]. Lopinavir may thus foster apoptosis and inhibit growth of tumor cells [15-18].

Oxidative stress may similarly trigger eryptosis, the suicidal death of erythrocytes, characterized by cell shrinkage [19] and phospholipid scrambling of the cell membrane, which is apparent from phosphatidylserine translocation to the cell surface [20]. Oxidative stress is in part effective by opening of Ca^{2+} permeable unselective cation channels with subsequent Ca^{2+} entry and increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [20]. Eryptosis is further triggered by ceramide [21], energy depletion [20], and caspases [20, 22, 23]. Eryptosis is stimulated by several kinases including casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and p38 kinase [20]. Eryptosis is inhibited by AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase, and sorafenib/sunitinib sensitive kinases [20]. Triggers of eryptosis further include a wide variety of xenobiotics [20, 24-49]. Enhanced eryptosis is observed in several clinical conditions, such as chronic kidney disease (CKD) [29, 50-52], hemolytic-uremic syndrome [53], sepsis [54], dehydration [37], hyperphosphatemia [47], hepatic failure [55], diabetes [56], malignancy [20], malaria [20, 57-59], sickle-cell disease [20], beta-thalassemia [20], Hb-C and G6PD-deficiency [20], as well as Wilsons disease [60].

The present study explored whether lopinavir triggers eryptosis. To this end, human erythrocytes from healthy volunteers were exposed for 48 hours to lopinavir and phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$, reactive oxygen species, GSH abundance 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 for 48 hours *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. Where indicated, erythrocytes were exposed to lopinavir (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 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 lopinavir 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

In order to determine hemolysis, the samples were centrifuged (3 min at 1600 rpm, room temperature) after incubation under the respective experimental conditions and the supernatants were harvested. As a measure of hemolysis, the haemoglobin (Hb) concentration of the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100 % hemolysis. Hemolysis is expressed in % in order to allow comparison with % annexin V binding cells.

Intracellular Ca²⁺

After incubation, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl₂ 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 CaCl₂. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer solution. Then, Ca²⁺-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 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 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).

GSH abundance

The content of reduced glutathione was measured using mercury orange. After incubation, erythrocytes were washed in Ringer solution and then loaded with 40 μM mercury orange (Sigma-Aldrich, Hamburg, Germany) in PBS and incubated at 37°C for 3 mins. Afterwards, the samples were washed once and were finally resuspended in 200 μl PBS. The fluorescence intensity was measured in FL-2 channel by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 576 nm.

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 μg/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.

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 tested whether lopinavir stimulates suicidal erythrocyte death or eryptosis. The two hallmarks of eryptosis are cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface. Annexin-V-binding was determined by flow cytometry as a measure of phosphatidylserine exposure at the erythrocyte surface. The measurements were made following incubation for 48 hours in Ringer solution without or with lopinavir (5 - 20 μg/ml). As illustrated in Fig. 1, a 48 hours exposure to lopinavir was followed by an increase of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 10 μg/ml lopinavir.

In order to identify shrunken erythrocytes, erythrocyte volume was estimated from forward scatter, which was determined utilizing flow cytometry. Again, the measurements

Fig. 1. Effect of lopinavir 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 20 µg/ml lopinavir. B. Arithmetic means $\pm$ SEM ($n = 12$) of erythrocyte annexin-V-binding (black bars) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of lopinavir (5 - 20 µg/ml). For comparison, the effect of the solvent DMSO (grey bar) is provided. **($p < 0.01$), ***($p < 0.001$) indicate significant difference from the absence of lopinavir (ANOVA).

Fig. 2. Effect of lopinavir 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 20 µg/ml lopinavir. B. Arithmetic means $\pm$ SEM ($n = 12$) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of lopinavir (5 - 20 µg/ml). For comparison, the effect of the solvent DMSO (grey bar) is provided. **($p < 0.01$) indicates significant difference from the absence of lopinavir (ANOVA).

were made following a 48 hours incubation in Ringer solution without or with lopinavir (5 - 20 µg/ml). As apparent from Fig. 2, lopinavir treatment was followed by a decrease of erythrocyte forward scatter, an effect reaching statistical significance at 15 µg/ml lopinavir concentration.

In order to estimate the effect of lopinavir on hemolysis, the haemoglobin concentration in the supernatant was determined. As a result, following a 48 hours incubation the percentage hemolysed erythrocytes increased from 1.9 ± 0.2 % ($n = 4$) in the absence of lopinavir to 3.6 ± 0.4 % (5 µg/ml), 7.9 ± 1.8 % (10 µg/ml), 15.7 ± 2.2 % (15 µg/ml) and 28.8 ± 6.7 % (20 µg/ml) in the presence of lopinavir ($n = 4$ each). The effect reached statistical significance at 15 µg/ml lopinavir concentration.

Cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) was estimated utilizing Fluo3 fluorescence. As illustrated in Fig. 3, a 48 hours exposure to lopinavir was followed by an increase of the Fluo3 fluorescence, an effect reaching statistical significance at 20 µg/ml lopinavir concentration.

In order to test whether the effect of lopinavir on phosphatidylserine translocation required entry of extracellular Ca^{2+} , erythrocytes were incubated for 48 hours in the absence or presence of 20 µg/ml lopinavir in the presence or nominal absence of extracellular Ca^{2+} . As apparent from Fig. 4, removal of extracellular Ca^{2+} significantly blunted the effect of lopinavir on annexin-V-binding. However, even in the absence of extracellular Ca^{2+} , lopinavir significantly increased the percentage of annexin-V-binding erythrocytes. Thus, lopinavir

Fig. 3. Effect of lopinavir 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 20 $\mu\text{g}/\text{ml}$ lopinavir. B. Arithmetic means $\pm$ SEM ($n = 12$) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) presence of lopinavir (5 – 20 $\mu\text{g}/\text{ml}$). For comparison, the effect of the solvent DMSO (grey bar) is provided. $^{*}(p < 0.05)$ indicates significant difference from the absence of lopinavir (ANOVA).

Fig. 4. Ca^{2+} sensitivity of lopinavir -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) presence of 20 $\mu\text{g}/\text{ml}$ lopinavir in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means $\pm$ SEM ($n = 11$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) presence of 20 $\mu\text{g}/\text{ml}$ lopinavir (20 $\mu\text{g}/\text{ml}$) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . $^{***}(p < 0.001)$ indicates significant difference from the absence of lopinavir; $^{##}(p < 0.01)$ indicates significant difference from the presence of Ca^{2+} (ANOVA).

triggered erythrocyte cell membrane scrambling in part but not exclusively by stimulating entry of extracellular Ca^{2+} .

Stimulators of Ca^{2+} entry and eryptosis include oxidative stress. DCFDA (2',7'-dichlorodihydrofluorescein diacetate) was thus employed to quantify reactive oxygen species (ROS). As illustrated in Fig. 5, a 48 hours exposure to lopinavir (20 $\mu\text{g}/\text{ml}$) was followed by a significant increase of DCFDA fluorescence. Accordingly, lopinavir induced oxidative stress.

Fig. 5. Effect of lopinavir 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 20 µg/ml lopinavir. B. Arithmetic means $\pm$ SEM ($n = 8$) of the DCFDA fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) presence of 20 µg/ml lopinavir. **($p < 0.01$) indicates significant difference from the absence of lopinavir (paired t test).

Fig. 6. Effect of lopinavir on erythrocyte GSH level. A. Original histogram of mercury orange fluorescence of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 20 µg/ml lopinavir. B. Arithmetic means $\pm$ SEM ($n = 16$) of mercury orange fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) presence of 20 µg/ml lopinavir. *($p < 0.05$) indicates significant difference from the absence of lopinavir (paired t test).

The enhanced oxidative stress could have resulted from decreased GSH levels leading to an impaired anti-oxidative stress defense. Thus, mercury orange staining was employed to quantify GSH abundance. As illustrated in Fig. 6, the mercury orange-dependent fluorescence was significantly lower after exposure to 20 µg/ml lopinavir for 48 hours compared to exposure to Ringer solution.

Additional experiments addressed the impact of lopinavir on ceramide abundance at the erythrocyte surface. The ceramide abundance was quantified utilizing specific antibodies. As a result, following a 48 hours incubation, the ceramide abundance was similar following incubation with 20 µg/ml lopinavir (15.6 ± 0.5 a.u., $n = 11$) and in the absence of lopinavir (15.7 ± 0.4 a.u., $n = 11$).

Discussion

The present observations unravel a novel effect of lopinavir, i.e. the stimulation of eryptosis, the suicidal erythrocyte death. Exposure of human erythrocytes drawn from healthy individuals to lopinavir is followed by cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the erythrocyte surface. The effect of lopinavir on eryptosis was accompanied by enhanced hemolysis. The concentrations required for the effect are well in the range of plasma concentrations determined in patients under lopinavir treatment [61]. The observed eryptosis and hemolysis may thus well explain the anemia following lopinavir treatment [14].

The effect of lopinavir on cell membrane scrambling was obviously in large part due to increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) resulting from Ca^{2+} entry from the extracellular space. Accordingly, removal of extracellular Ca^{2+} significantly blunted the lopinavir-induced cell membrane scrambling. Ca^{2+} entered presumably through Ca^{2+} permeable cation channels, which are known to be activated by oxidative stress [20]. As apparent from the increase of DCFDA fluorescence, lopinavir treatment did increase the abundance of reactive oxygen species. The increase in oxidative stress was paralleled by a decreased GSH abundance.

The increase of $[\text{Ca}^{2+}]_i$ presumably further accounts for the observed lopinavir-induced erythrocyte shrinkage, as an increase of $[\text{Ca}^{2+}]_i$ leads to activation of 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 [19].

The stimulation of eryptosis by lopinavir may lead to anemia, as long as the formation of new erythrocytes cannot match the loss of eryptotic erythrocytes [20]. Moreover, phosphatidylserine exposing erythrocytes adhere to the vascular wall [62], stimulate blood clotting, and trigger thrombosis [63-65]. The stimulation of eryptosis by lopinavir may thus compromise microcirculation [21, 63, 66-69].

In conclusion, lopinavir 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.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Tanja Loch. The study was supported by the Deutsche Forschungsgemeinschaft.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

References

- 1 Barragan P, Podzamczar D: Lopinavir/ritonavir: a protease inhibitor for HIV-1 treatment. *Expert Opin Pharmacother* 2008;9:2363-2375.
- 2 Caswell RJ, Phillips D, Chaponda M, Khoo SH, Taylor GP, Ghanem M, Poulton M, Welch J, Gibbons S, Jackson V, Lambert JS: Utility of therapeutic drug monitoring in the management of HIV-infected pregnant women in receipt of lopinavir. *Int J STD AIDS* 2011;22:11-14.
- 3 Chandwani A, Shuter J: Lopinavir/ritonavir in the treatment of HIV-1 infection: a review. *Ther Clin Risk Manag* 2008;4:1023-1033.
- 4 Chetchotisakd P: The CASTLE study: atazanavir/r versus lopinavir/r in antiretroviral-naïve patients. *Expert Rev Anti Infect Ther* 2009;7:801-805.
- 5 Croxtall JD, Perry CM: Lopinavir/Ritonavir: a review of its use in the management of HIV-1 infection. *Drugs* 2010;70:1885-1915.

- 6 Marchetti G, Merlini E, Sinigaglia E, Iannotti N, Bai F, Savoldi A, Tincati C, Carpani G, Bini T, Arminio Monforte A: Immune reconstitution in HIV+ subjects on lopinavir/ritonavir-based HAART according to the severity of pre-therapy CD4+. *Curr HIV Res* 2012;10:597-605.
- 7 Martinez BL, Riordan FA: Novel strategies in the use of lopinavir/ritonavir for the treatment of HIV infection in children. *HIV AIDS (Auckl)* 2010;2:59-67.
- 8 Moultrie H, McIlleron H, Sawry S, Kellermann T, Wiesner L, Kindra G, Gous H, Van Rie A: Pharmacokinetics and safety of rifabutin in young HIV-infected children receiving rifabutin and lopinavir/ritonavir. *J Antimicrob Chemother* 2015;70:543-549.
- 9 Murphy RA, Marconi VC, Gandhi RT, Kuritzkes DR, Sunpath H: Coadministration of lopinavir/ritonavir and rifampicin in HIV and tuberculosis co-infected adults in South Africa. *PLoS One* 2012;7:e44793.
- 10 Pasley MV, Martinez M, Hermes A, d'Amico R, Nilius A: Safety and efficacy of lopinavir/ritonavir during pregnancy: a systematic review. *AIDS Rev* 2013;15:38-48.
- 11 Perry M, Taylor GP, Sabin CA, Conway K, Flanagan S, Dwyer E, Stevenson J, Mulka L, McKendry A, Williams E, Barbour A, Dermont S, Roedling S, Shah R, Anderson J, Rodgers M, Wood C, Sarner L, Hay P, Hawkins D, deRuiter A: Lopinavir and atazanavir in pregnancy: comparable infant outcomes, virological efficacies and preterm delivery rates. *HIV Med* 2015;10.1111/hiv.12277
- 12 Pitrak DL, Estes R, Novak RM, Linares-Diaz M, Tschampa JM: Beneficial effects of a switch to a Lopinavir/ritonavir-containing regimen for patients with partial or no immune reconstitution with highly active antiretroviral therapy despite complete viral suppression. *AIDS Res Hum Retroviruses* 2011;27:659-667.
- 13 Torres B, Rallon NI, Lonca M, Diaz A, Alos L, Martinez E, Cruceta A, Arnaiz JA, Leal L, Lucero C, Leon A, Sanchez M, Negro E, Clotet B, Gatell JM, Benito JM, Garcia F: Immunological function restoration with lopinavir/ritonavir versus efavirenz containing regimens in HIV-infected patients: a randomized clinical trial. *AIDS Res Hum Retroviruses* 2014;30:425-433.
- 14 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.
- 15 Kraus M, Muller-Ide H, Ruckrich T, Bader J, Overkleeft H, Driessen C: Ritonavir, nelfinavir, saquinavir and lopinavir induce proteotoxic stress in acute myeloid leukemia cells and sensitize them for proteasome inhibitor treatment at low micromolar drug concentrations. *Leuk Res* 2014;38:383-392.
- 16 Taura M, Kariya R, Kudo E, Goto H, Iwawaki T, Amano M, Suico MA, Kai H, Mitsuya H, Okada S: Comparative analysis of ER stress response into HIV protease inhibitors: lopinavir but not darunavir induces potent ER stress response via ROS/JNK pathway. *Free Radic Biol Med* 2013;65:778-788.
- 17 Kariya R, Taura M, Suzu S, Kai H, Katano H, Okada S: HIV protease inhibitor Lopinavir induces apoptosis of primary effusion lymphoma cells via suppression of NF-kappaB pathway. *Cancer Lett* 2014;342:52-59.
- 18 Johnson MD, O'Connell M, Pilcher W: Lopinavir inhibits meningioma cell proliferation by Akt independent mechanism. *J Neurooncol* 2011;101:441-448.
- 19 Lang PA, Kaiser S, Myssina S, Wiedner T, Lang F, Huber SM: Role of Ca²⁺-activated K⁺ channels in human erythrocyte apoptosis. *Am J Physiol Cell Physiol* 2003;285:C1553-C1560.
- 20 Lang F, Qadri SM: Mechanisms and significance of eryptosis, the suicidal death of erythrocytes. *Blood Purif* 2012;33:125-130.
- 21 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.
- 22 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.
- 23 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.
- 24 Jilani K, Lang F: Carmustine-induced phosphatidylserine translocation in the erythrocyte membrane. *Toxins (Basel)* 2013;5:703-716.
- 25 Vota DM, Maltaner 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.

- 26 Zappulla D: Environmental stress, erythrocyte dysfunctions, inflammation, and the metabolic syndrome: adaptations to CO₂ increases? *J Cardiometa Syndr* 2008;3:30-34.
- 27 Lupescu A, Jilani K, Zbidah M, Lang F: Patulin-induced suicidal erythrocyte death. *Cell Physiol Biochem* 2013;32:291-299.
- 28 Abed M, Zoubi KA, Theurer M, Lang F: Effect of dermaseptin on erythrocytes. *Basic Clin Pharmacol Toxicol* 2013;113:347-352.
- 29 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.
- 30 Ghashghaieina 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.
- 31 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.
- 32 Jilani K, Enkel S, Bissinger R, Almilaji A, Abed M, Lang F: Fluoxetine induced suicidal erythrocyte death. *Toxins (Basel)* 2013;5:1230-1243.
- 33 Lupescu A, Bissinger R, Jilani K, Lang F: Triggering of suicidal erythrocyte death by celecoxib. *Toxins (Basel)* 2013;5:1543-1554.
- 34 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.
- 35 Oswald G, Alzoubi K, Abed M, Lang F: Stimulation of suicidal erythrocyte death by ribavirin. *Basic Clin Pharmacol Toxicol* 2014;114:311-317.
- 36 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.
- 37 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.
- 38 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.
- 39 Arnold M, Bissinger R, Lang F: Mitoxantrone-induced suicidal erythrocyte death. *Cell Physiol Biochem* 2014;34:1756-1767.
- 40 Bissinger R, Fischer S, Jilani K, Lang F: Stimulation of Erythrocyte Death by Phloretin. *Cell Physiol Biochem* 2014;34:2256-2265.
- 41 Bissinger R, Lupescu A, Zelenak C, Jilani K, Lang F: Stimulation of eryptosis by cryptotanshinone. *Cell Physiol Biochem* 2014;34:432-442.
- 42 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.
- 43 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.
- 44 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.
- 45 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.
- 46 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.
- 47 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.
- 48 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.
- 49 Pagano M, Faggio C: The use of erythrocyte fragility to assess xenobiotic cytotoxicity. *Cell Biochem Funct* 2015;33:351-355.
- 50 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.

- 51 Polak-Jonkisz D, Purzyc L: Ca(2+) influx versus efflux during eryptosis in uremic erythrocytes. *Blood Purif* 2012;34:209-210; author reply 210.
- 52 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.
- 53 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.
- 54 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.
- 55 Lang E, Gatidis S, Freise NF, Bock H, Kubitz R, Lauermaann C, Orth HM, Klindt C, Schuier M, Keitel V, Reich M, Liu G, Schmidt S, Xu HC, Qadri SM, Herebian D, Pandya 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.
- 56 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.
- 57 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.
- 58 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.
- 59 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.
- 60 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, Heftner 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.
- 61 Moholisa RR, Schomaker M, Kuhn L, Meredith S, Wiesner L, Coovadia A, Strehlau R, Martens L, Abrams EJ, Maartens G, McIlerron H: Plasma lopinavir concentrations predict virological failure in a cohort of South African children initiating a protease-inhibitor-based regimen. *Antivir Ther* 2014;19:399-406.
- 62 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.
- 63 Andrews DA, Low PS: Role of red blood cells in thrombosis. *Curr Opin Hematol* 1999;6:76-82.
- 64 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.
- 65 Zwaal RF, Comfurius P, Bevers EM: Surface exposure of phosphatidylserine in pathological cells. *Cell Mol Life Sci* 2005;62:971-988.
- 66 Closse C, Dachary-Prigent J, Boisseau MR: Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. *Br J Haematol* 1999;107:300-302.
- 67 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.
- 68 Pandolfi A, Di Pietro N, Siroli 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.
- 69 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.