Original Paper

Clofazimine Induced Suicidal Death of Human Erythrocytes

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Key Words

Phosphatidylserine • Ca^{2+} • Phospholipase A_2 • ATP • Eryptosis

Abstract

Background/Aims: The antimycobacterial riminophenazine clofazimine has previously been shown to up-regulate cellular phospholipase A_2 and to induce apoptosis. In erythrocytes phospholipase A_2 stimulates eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Phospholipase A_2 is in part effective by fostering formation of prostaglandin E_2 , which triggers Ca^{2+} entry. Stimulators of Ca^{2+} entry and eryptosis further include oxidative stress and energy depletion. The present study tested, whether and how clofazimine induces eryptosis. **Methods:** Phosphatidylserine exposure at the cell surface was estimated from annexin V binding, cell volume from forward scatter, hemolysis from hemoglobin release, cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) from Fluo3-fluorescence, reactive oxygen species (ROS) from 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence, and cytosolic ATP level utilizing a luciferin-luciferase assay kit. **Results:** A 24-48 hours exposure of human erythrocytes to clofazimine ($\geq 1.5 \mu\text{g/ml}$) significantly increased the percentage of annexin-V-binding cells without appreciably modifying forward scatter. Clofazimine significantly increased $[\text{Ca}^{2+}]_i$, significantly decreased cytosolic ATP, but did not significantly modify ROS. The effect of clofazimine on annexin-V-binding was significantly blunted, but not fully abolished by removal of extracellular Ca^{2+} , and by phospholipase A_2 inhibitor quinacrine (25 μM). Clofazimine further augmented the effect of Ca^{2+} ionophore ionomycin (0.1 μM) on eryptosis. The clofazimine induced annexin-V-binding was, however, completely abrogated by combined Ca^{2+} removal and addition of quinacrine. **Conclusion:** Clofazimine stimulates phospholipid scrambling of the erythrocyte cell membrane, an effect in part dependent on entry of extracellular Ca^{2+} , paralleled by cellular energy depletion and sensitive to phospholipase A_2 inhibitor quinacrine.

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Introduction

The riminophenazine antibiotic clofazimine is used for the treatment of mycobacterial infections including but not limited to leprosy and drug resistant tuberculosis [1-13]. Clofazimine has further anti-inflammatory potency and is considered for the treatment of

several inflammatory disorders including psoriasis and autoimmune disease [1, 4, 6, 8, 14]. Its use is, however, limited by its toxicity [15].

Clofazimine may create intracellular crystal-like drug inclusions [16] and intercalate into bacterial DNA [1]. Clofazimine-sensitive cellular mechanisms further include mitochondrial depolarisation [16], up-regulation of cellular phospholipase A₂ [1], caspase activation [17], and induction of apoptosis [16, 18].

Phospholipase A₂ activity with subsequent formation of prostaglandin E₂ [19] may lead to eryptosis, the suicidal death of erythrocytes characterized by cell shrinkage [20] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [21]. PGE₂ is effective by stimulating Ca²⁺ entry with increase of cytosolic Ca²⁺ activity ([Ca²⁺]_i) [21]. Ca²⁺ entry may, in addition, be triggered by oxidative stress [21]. Further cellular mechanisms fostering eryptosis include ceramide [22], energy depletion [21], as well as activated caspases [21, 23, 24], casein kinase 1α, 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, and sorafenib/sunitinib sensitive kinases [21]. A wide variety of xenobiotics have been shown to activate or inhibit eryptosis [21, 25-53].

The present study explored, whether and, if so, how clofazimine triggers eryptosis. To this end, human erythrocytes from healthy volunteers were treated with clofazimine and phosphatidylserine surface abundance and cell volume 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 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 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl₂; pH 7.4 at 37°C for 24 h. In Ca²⁺ free solutions, Ca²⁺ was removed without replacement. The high buffer capacity (32 HEPES) was used in order to prevent appreciable pH changes despite lactate formation by erythrocytes. Where indicated, erythrocytes were exposed to clofazimine (Sigma Aldrich, Hamburg, Germany), or quinacrine (Sigma Aldrich, Hamburg, Germany) at the indicated concentrations.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 150 µl cell suspension was washed in Ringer solution containing 5 mM CaCl₂ and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 20 min under protection from light. The annexin V abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). 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

For the determination of hemolysis, the samples were centrifuged (10 min at 2000 rpm, room temperature) after incubation under the respective experimental conditions and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (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.

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 µM Fluo-3/AM. The cells were incubated at 37°C for 30 min and washed twice in Ringer solution containing 5 mM CaCl₂. The Fluo-3/AM-loaded erythrocytes

were resuspended in 200 μ l 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 oxygen species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 100 μ l 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 μ M. Erythrocytes were incubated at 37 °C for 30 min in the dark and then washed three times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 μ l 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).

Intracellular ATP levels

For the determination of intracellular erythrocyte ATP, 80 μ l of erythrocyte pellets were incubated for 24 h at 37 °C in Ringer solution with or without clofazimine (1.5 μ g/ml). All subsequent manipulations were performed at 4 °C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of HClO_4 (5%). After centrifugation (Eppendorf Microcentrifuge 5417R, Hamburg, Germany) at 2000 rpm for 5 min the erythrocytes were washed once in PBS and centrifuged again. An aliquot of the supernatant (400 μ l) was adjusted to pH 7.7 by addition of saturated KHCO_3 solution. After dilution of the supernatant, the ATP concentrations of the aliquots were determined utilizing a luciferin-luciferase assay kit (Roche Diagnostics) on a luminometer (Berthold Biolumat LB9500, Bad Wildbad, Germany) according to the manufacturer's protocol.

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 explored the effect of clofazimine on eryptosis, the suicidal erythrocyte death. The most important hallmark of eryptosis is cell membrane scrambling with phosphatidylserine translocation to the cell surface. Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. The erythrocytes were analysed following incubation for 24 or 48 hours in Ringer solution without or with clofazimine (1.5 – 7.5 μ g/ml). As shown in Fig. 1 exposure to clofazimine increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 7.5 μ g/ml after 24 hours and at 1.5 μ g/ml after 48 hours.

In order to quantify hemolysis, the hemoglobin concentration in the supernatant was determined by photometry. As shown in Fig. 1D, a 48 hours incubation with clofazimine (1.5 – 7.5 μ g/ml) slightly increased hemolysis, an effect reaching statistical significance at 7.5 μ g/ml.

Eryptosis is typically paralleled by cell shrinkage. In order to estimate cell volume, forward scatter was determined utilizing flow cytometry. As a result, the forward scatter was similar following a 24 hours incubation without (461 ± 12 , *n* = 4) and with 1.5 μ g/ml (461 ± 15 , *n* = 4) or 7.5 μ g/ml (462 ± 16 , *n* = 4) clofazimine. Moreover, the forward scatter was similar following a 48 hours incubation without (446 ± 9 , *n* = 4) and with 1.5 μ g/ml (461 ± 10 , *n* = 4) or 7.5 μ g/ml (453 ± 9 , *n* = 4) clofazimine.

In order to determine whether clofazimine modifies cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), Fluo3 fluorescence was taken as measure of $[\text{Ca}^{2+}]_i$. As illustrated in Fig. 2, a 48 hours exposure to clofazimine (1.5 and 7.5 μ g/ml) significantly increased the Fluo3 fluorescence.

Fig. 1. Effect of clofazimine on phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 24 hours (A) or 48 hours (B) to Ringer solution without (grey areas) and with (black lines) presence of 1.5 $\mu\text{g/ml}$ clofazimine. C,D. Arithmetic means $\pm$ SEM ($n = 4$) of erythrocyte annexin-V-binding following incubation for 24 hours (C) or 48 hours (D) to Ringer solution without (white bars) or with (black bars) presence of clofazimine (1.5 – 7.5 $\mu\text{g/ml}$). For comparison, the effect of clofazimine on hemolysis is shown (grey bars). ***($p < 0.001$), #($p < 0.05$) indicates significant difference from the absence of clofazimine (ANOVA).

Fig. 2. Effect of clofazimine 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 clofazimine (1.5 $\mu\text{g/ml}$). (B). Arithmetic means $\pm$ SEM ($n = 4$) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) clofazimine (1.5 – 7.5 $\mu\text{g/ml}$). *($p < 0.05$) indicates significant difference from the absence of clofazimine (ANOVA).

Fig. 3. Ca^{2+} sensitivity of clofazimine-induced phosphatidylserine exposure. Arithmetic means $\pm$ SEM ($n = 4$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (grey and black bars) clofazimine (1.5 – 7.5 $\mu\text{g}/\text{ml}$) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . * ($P < 0.05$), *** ($P < 0.001$) indicates significant difference from the absence of clofazimine, ### ($p < 0.001$) indicates significant difference from corresponding value the presence of Ca^{2+} (ANOVA).

Fig. 4. Effect of clofazimine and Ca^{2+} ionophore ionomycin on phosphatidylserine exposure. (A) Arithmetic means $\pm$ SEM of erythrocyte annexin-V-binding ($n = 8$) following incubation for 24 hours to Ringer solution without (white bars) or with (black bars) presence of clofazimine (7.5 $\mu\text{g}/\text{ml}$) in the absence (left bars) and presence (right bars, ionomycin) of calcium ionophore ionomycin (0.1 μM). (B) Arithmetic means $\pm$ SEM of erythrocyte forward scatter ($n = 8$) following incubation for 24 hours to Ringer solution without (white bars) or with (black bars) presence of clofazimine (7.5 $\mu\text{g}/\text{ml}$) in the absence (left bars) and presence (right bars, ionomycin) of calcium ionophore ionomycin (0.1 μM). ** ($p < 0.01$), *** ($p < 0.001$) indicates significant difference from the absence of clofazimine, # ($p < 0.05$), ## ($p < 0.01$) indicates significant difference from the absence of ionomycin (ANOVA).

A further series of experiments explored whether clofazimine-induced translocation of phosphatidylserine to the cell surface required entry of extracellular Ca^{2+} . To this end, erythrocytes were incubated for 48 hours in the absence or presence of 1.5 or 7.5 $\mu\text{g}/\text{ml}$ clofazimine, both in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 3, removal of extracellular Ca^{2+} significantly blunted the effect of clofazimine on annexin-V-binding. Nevertheless, even in the absence of extracellular Ca^{2+} clofazimine significantly increased the percentage of annexin-V-binding erythrocytes. Thus, eryptosis was in large part but not fully triggered by entry of extracellular Ca^{2+} .

Additional experiments explored whether clofazimine was effective even in erythrocytes exposed to Ca^{2+} ionophore ionomycin. To this end, erythrocytes were exposed for 24 hours to 7.5 $\mu\text{g}/\text{ml}$ clofazimine in the absence or presence of ionomycin (0.1 μM). As illustrated in Fig. 4, clofazimine significantly augmented the effect of ionomycin on annexin-V-binding and forward scatter, an observation again pointing to an additional mechanism involved in the triggering of eryptosis by clofazimine.

Fig. 5. Effect of Clofazimine on phosphatidylserine exposure in absence and presence of phospholipase A₂ inhibitor quinacrine. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution in the absence (grey area) and presence (black line) of 1.5 µg/ml clofazimine in the absence (A) and presence (B) of phospholipase A₂ inhibitor quinacrine (25 µM). (C) Arithmetic means ± SEM of erythrocyte annexin-V-binding (n = 8) following incubation for 24 hours to Ringer solution without (white bars) or with (black bars) presence of clofazimine (5 µg/ml) in the absence (left bars) and presence (right bars) of phospholipase A₂ inhibitor quinacrine (25 µM). *** (p<0.001) indicates significant difference from the absence of clofazimine, # (p<0.05) indicates significant difference from the absence of quinacrine (ANOVA).

Fig. 6. Effect of simultaneous Ca²⁺ removal and addition of quinacrine on clofazimine-induced phosphatidylserine exposure. Arithmetic means ± SEM (n = 4) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) clofazimine (7.5 µg/ml) in the presence (left bars), and absence (middle and right bars, -Ca²⁺) of Ca²⁺ and the absence (left and middle bars) and presence (right bars, -Ca²⁺ + quinacrine) of 25 µM quinacrine. * (p<0.05), *** (p<0.001) indicates significant difference from the absence of clofazimine, ### (p<0.001) indicates significant difference from corresponding value the presence of Ca²⁺ and absence of quinacrine (ANOVA).

Fig. 7. Effect of clofazimine on erythrocyte ATP levels. Arithmetic means ± SEM (n = 8) of cellular ATP (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) clofazimine (1.5 µg/ml). ** (p<0.01) indicates significant difference from the absence of clofazimine (t-test).

Triggers of eryptosis include oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As a result, the DCFDA fluorescence was similar following a 48 hours incubation without (11.0 ± 1.8 , n = 4) and with 7.5 µg/ml (11.2 ± 0.6 , n = 4) clofazimine. In contrast, the fluorescence was significantly increased (62.2 ± 3.6 , n = 4) following treatment with the oxidant tert-butylhydroperoxide (tBOOH) as a positive control.

Additional experiments explored whether clofazimine-induced cell membrane scrambling was dependent on activation of phospholipase A₂. To this end, erythrocytes were exposed for 48 hours to 1.5 µg/ml clofazimine in the absence or presence of the unselective phospholipase A₂ inhibitor quinacrine (25 µM). As illustrated in Fig. 5, the effect of clofazimine on annexin-V-binding was significantly blunted in the presence of phospholipase A₂ inhibitor quinacrine (25 µM).

To test whether clofazimine-induced cell membrane scrambling was abolished by the combination of Ca²⁺ removal and addition of quinacrine, erythrocytes were exposed for 48 hours to 7.5 µg/ml clofazimine in the presence of Calcium, absence of calcium, or the combination of calcium absence and quinacrine presence. As illustrated in Fig. 6, the effect of clofazimine on annexin-V-binding was fully abolished by combined Ca²⁺ removal and quinacrine addition.

Eryptosis is further stimulated by energy depletion. A luciferin-luciferase assay was thus employed to determine the cytosolic ATP level. As illustrated in Fig. 7, a 48 hours exposure to 1.5 µg/ml clofazimine significantly decreased the cytosolic ATP level.

Discussion

The present observations disclose a novel effect of the riminophenazine clofazimine, i.e. the triggering of phospholipid scrambling of the erythrocyte cell membrane, a key event in suicidal erythrocyte death or eryptosis. The clofazimine concentration (1.5 µg/ml) required for stimulation of erythrocyte cell membrane scrambling was within the range of concentrations observed *in vivo* [4, 54]. It must be kept in mind that the sensitivity of erythrocyte cell membrane scrambling to clofazimine may be enhanced in several clinical conditions associated with enhanced susceptibility to eryptosis, such as dehydration [41], hyperphosphatemia [52] chronic kidney disease (CKD) [30, 55-57], hemolytic-uremic syndrome [58], diabetes [59], liver failure [60], malignancy [61], sepsis [62] and Wilsons disease [63].

The effect of clofazimine on cell membrane scrambling was paralleled by an increase of cytosolic Ca²⁺ activity ([Ca²⁺]_i). More importantly, the effect of clofazimine on cell membrane scrambling was strongly blunted in the absence of extracellular Ca²⁺ and thus depended largely on Ca²⁺ entry. Nevertheless, even in the absence of extracellular Ca²⁺ clofazimine still increased cell membrane scrambling, an observation pointing to some additional mechanism.

Even though clofazimine increased [Ca²⁺]_i, it did not appreciably influence cell volume. An increase of [Ca²⁺]_i were expected to activate Ca²⁺ sensitive K⁺ channels with subsequent cell shrinkage due to K⁺ exit, cell membrane hyperpolarization, Cl⁻ exit and thus cellular loss of KCl with water [20]. It remains elusive, why clofazimine was without effect on cell volume.

The stimulation of cell membrane scrambling was further not paralleled by appreciable increases of reactive oxygen species (ROS), a known trigger of Ca²⁺ entry and subsequent cell membrane scrambling [21].

The clofazimine induced eryptosis was significantly blunted by phospholipase A₂ inhibitor quinacrine [21]. Clofazimine has previously been shown to up-regulate phospholipase A2 in nucleated cells [1]. The combined Ca²⁺ removal and quinacrine addition fully abrogated the effect of clofazimine, indicating that the two mechanisms account for most of the stimulation of eryptosis by clofazimine.

Clofazimine further moderately decreased cellular ATP, an effect presumably contributing to the stimulation of cell membrane scrambling. Energy depletion is a well known stimulator of eryptosis [21].

Phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood, a mechanism ensuring disposal of defective erythrocytes prior to hemolysis [21]. Eryptosis thus prevents release of hemoglobin, which would otherwise be filtered in renal glomerula, precipitate in tubular fluid and thus occlude nephrons [64]. In malaria, *Plasmodium*-infected erythrocytes similarly expose phosphatidylserine, which fosters their clearance from circulating blood [21]. Triggering of cell membrane scrambling in infected erythrocytes may

thus favourably influence the clinical course of malaria [65]. Sick cell trait, beta-thalassemia-trait, Hb-C and G6PD-deficiency foster cell membrane scrambling of *Plasmodium*-infected erythrocytes and thus protect against a severe course of malaria [21, 66-68]. Iron deficiency [69], and treatment with lead [69], chlorpromazine [70] or NO synthase inhibitors [70] similarly sensitize erythrocytes to cell membrane scrambling and thus similarly counteract parasitemia. It is tempting to speculate that clofazimine may similarly foster cell membrane scrambling of *Plasmodium*-infected erythrocytes.

However, the accelerated clearance of phosphatidylserine exposing erythrocytes from circulating blood leads to anemia, as soon as the loss of erythrocytes outcasts the generation of new erythrocytes [21]. Moreover, phosphatidylserine exposing erythrocytes adhere to the vascular wall [71], trigger blood clotting and elicit thrombosis [72-74]. Stimulation of erythrocyte cell membrane scrambling may thus impair microcirculation [22, 72, 75-78].

Conclusion

Clofazimine triggers erythrocyte cell membrane scrambling. The effect is paralleled by and in part due to Ca^{2+} entry and energy depletion as well as abrogated by phospholipase A_2 inhibitor quinacrine.

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Disclosure Statement

The authors state that they have nothing to disclose.

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