

Original Paper

Stimulation of Suicidal Erythrocyte Death by the Antimalarial Drug Mefloquine

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Key Words

Phosphatidylserine • Calcium • Cell volume • ROS • Oxidative stress • Eryptosis • Malaria

Abstract

Background: The antimalarial drug mefloquine has previously been shown to stimulate apoptosis of nucleated cells. Similar to apoptosis, erythrocytes may enter suicidal death or eryptosis, which is characterized by cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane with phosphatidylserine translocation to the erythrocyte surface. Stimulators of eryptosis include oxidative stress, increase of cytosolic Ca^{2+} -activity ($[Ca^{2+}]_i$), and ceramide. **Methods:** Phosphatidylserine abundance at the cell surface was estimated from annexin V binding, cell volume from forward scatter, reactive oxidant species (ROS) from 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence, $[Ca^{2+}]_i$ from Fluo3-fluorescence, and ceramide abundance from specific antibody binding. **Results:** A 48 h treatment of human erythrocytes with mefloquine significantly increased the percentage of annexin-V-binding cells ($\geq 5 \mu\text{g/ml}$), significantly decreased forward scatter ($\geq 5 \mu\text{g/ml}$), significantly increased ROS abundance ($5 \mu\text{g/ml}$), significantly increased $[Ca^{2+}]_i$ ($7.5 \mu\text{g/ml}$) and significantly increased ceramide abundance ($10 \mu\text{g/ml}$). The up-regulation of annexin-V-binding following mefloquine treatment was significantly blunted but not abolished by removal of extracellular Ca^{2+} . Even in the absence of extracellular Ca^{2+} , mefloquine significantly increased annexin-V-binding. **Conclusions:** Mefloquine treatment leads to erythrocyte shrinkage and erythrocyte membrane scrambling, effects at least partially due to induction of oxidative stress, increase of $[Ca^{2+}]_i$ and up-regulation of ceramide abundance.

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Introduction

Mefloquine has been used for the chemoprophylaxis and treatment of malaria [1-8], schistosomes and other helminthes [9]. The use is limited by various side effects [4] including neuropsychiatric disorders [10-14], skin reactions [15] as well as risk of miscarriage and

stillbirth [16]. Mefloquine triggers apoptosis [17-21] and may sensitize tumor cells for cytostatic treatment [20]. Mechanisms involved in mefloquine induced apoptosis include oxidative stress [10, 18, 21], ceramide formation [22] and caspase activation [14, 18].

The clinical course of malaria could be favourably influenced by drugs stimulating suicidal erythrocyte death or eryptosis [23], which, similar to apoptosis, is characterized by cell shrinkage [24] and translocation of phosphatidylserine from the cell interior to the erythrocyte surface [25]. Stimulators of eryptosis include oxidative stress [25], increased cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), ceramide [26], energy depletion [25], and activated caspases [25, 27, 28]. Moreover, eryptosis is triggered by activated casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, p38 kinase, and PAK2 kinase [25], as well as by inhibited or lacking AMP activated kinase AMPK, cGMP-dependent protein kinase, sorafenib sensitive kinases and sunitinib sensitive kinases [25]. Stimulators of eryptosis further include diverse xenobiotics [25, 29-57].

Eryptotic phosphatidylserine exposing erythrocytes are engulfed by macrophages and thus rapidly cleared from circulating blood [25]. Accordingly, excessive eryptosis may lead to anemia, if the removal of eryptotic erythrocytes exceeds the formation of new erythrocytes [25]. Phosphatidylserine exposing erythrocytes may further bind to endothelial CXCL16/SR-PSO with adherence to the vascular wall [58]. They may further trigger blood clotting and thrombosis [59-61]. Thus, phosphatidylserine exposing erythrocytes may impair microcirculation [26, 59, 62-65].

The present study tested, whether and how mefloquine may trigger eryptosis. To this end, erythrocytes from healthy volunteers were incubated with or without mefloquine and phosphatidylserine and ceramide abundance at the erythrocyte surface, cell volume, reactive oxidant species and $[Ca^{2+}]_i$ determined utilizing 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 23 °C and the platelets and leukocytes-containing supernatant was disposed. Erythrocytes were incubated *in vitro* for 48 hours 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, and 1 CaCl₂; the pH was adjusted to 7.4 and the temperature kept at 37°C. Where indicated, erythrocytes were exposed to mefloquine (Sigma Aldrich, Hamburg, Germany), which was dissolved in DMSO (Carl Roth, Karlsruhe, Germany) and diluted to the indicated concentrations.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 150 μ l 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. Then annexin-V fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany). In addition, the forward scatter (FSC) of the cells was determined, which reflects cell volume but does not allow safe quantitative statements on percentage cell volume change.

Reactive oxidant species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 μ l suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA (Sigma, Schnellendorf, 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 Ca²⁺

After incubation, 150 µl cell suspension was 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 twice in Ringer solution containing 5 mM CaCl₂. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer. Then, Ca²⁺-dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Determination of ceramide abundance at the erythrocyte surface

To determine ceramide abundance, a monoclonal antibody-based assay 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 (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 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 explored whether mefloquine triggers eryptosis, the suicidal erythrocyte death. The hallmarks of eryptosis are phospholipid scrambling of the cell membrane and cell shrinkage.

Phospholipid scrambling of the cell membrane leads to phosphatidylserine translocation to the cell surface, which could be visualized with phosphatidylserine-binding FITC-labelled annexin-V. Annexin-V-binding was quantified utilizing flow cytometry. Prior to flow cytometry, the erythrocytes were incubated for 48 hours in Ringer solution without or with presence of mefloquine (1.25 -50 µg/ml). As shown in Fig. 1, a 48 h exposure to mefloquine increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 5 µg/ml mefloquine concentration. Mefloquine treatment is thus followed by phospholipid scrambling of the erythrocyte membrane with subsequent translocation of phosphatidylserine to the cell surface. An extended dose-response curve is provided in Fig. 1C, D.

As cell shrinkage is the second hallmark of eryptosis, erythrocyte cell volume was estimated from forward scatter in flow cytometry. Prior to flow cytometry, the erythrocytes were incubated for 48 hours in Ringer solution without or with mefloquine (1.25–50 µg/ml). As illustrated in Fig. 2, a 48 hours mefloquine treatment was followed by a decrease of erythrocyte forward scatter, an effect reaching statistical significance at 5 µg/ml mefloquine concentration.

Mechanisms triggering eryptosis include oxidative stress. Thus, additional experiments were performed to test, whether mefloquine influences the formation of reactive oxygen species (ROS). To this end, ROS was quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As shown in Fig. 3, a 48 hours exposure to mefloquine (1.25-10 µg/ml)

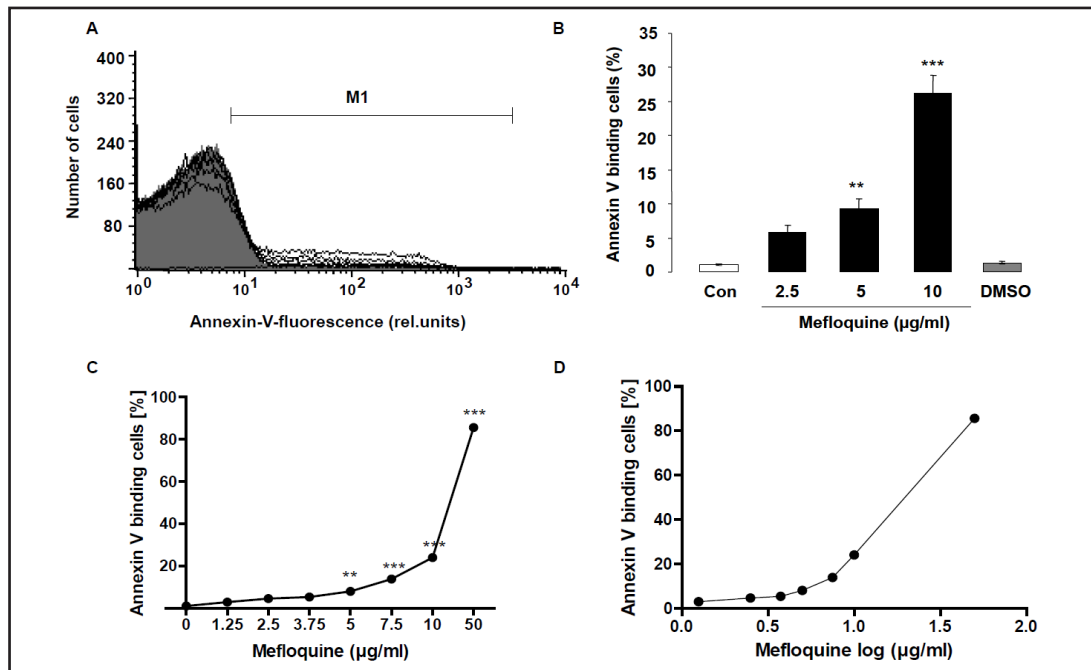
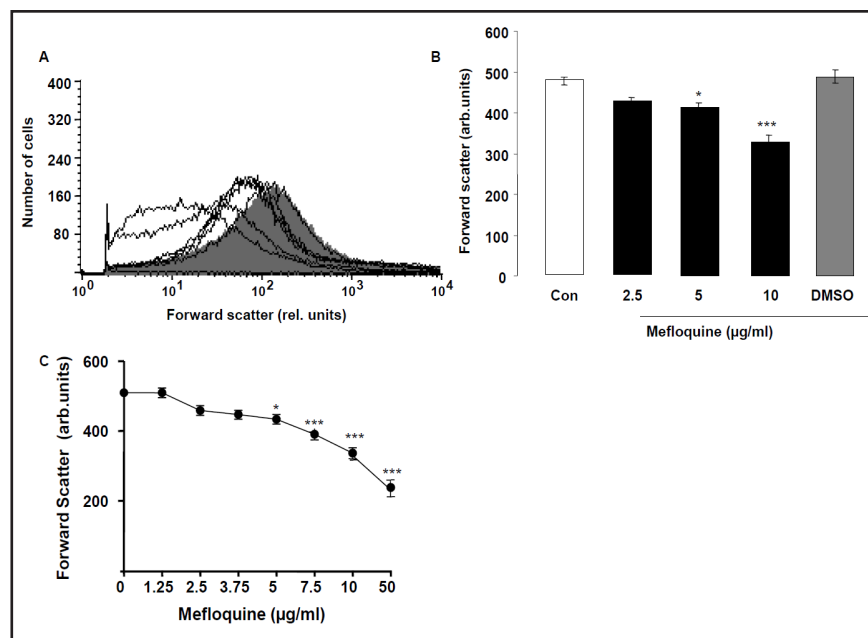


Fig. 1. Effect of mefloquine on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black lines) presence of 1.25-50 µg/ml mefloquine. M1 indicates the annexin-V-fluorescence defining the percentage of annexin-V-binding erythrocytes. B. Arithmetic means ± SEM of erythrocyte annexin-V-binding (n = 10) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of mefloquine (2.5 - 10 µg/ml). **(*P*<0.01), ***(*P*<0.001) indicate significant difference from the absence of mefloquine (ANOVA). C, D. Percentage of annexin V binding erythrocytes (%) as a linear (C) and semilogarithmic (D) function of mefloquine concentration (n=15). **(*P*<0.01), ***(*P*<0.001) indicate significant difference from the absence of mefloquine (ANOVA).

Fig. 2. Effect of mefloquine on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black lines) presence of 1.25-50 µg/ml mefloquine. B. Arithmetic means ± SEM (n = 10) of the erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) mefloquine (2.5 - 10 µg/ml).



or with (black bars) mefloquine (2.5 - 10 µg/ml). *(*P*<0.05), ***(*P*<0.001) indicate significant difference from the absence of mefloquine (ANOVA). C. Erythrocyte forward scatter as a function of mefloquine concentration (n=15). *(*P*<0.05), ***(*P*<0.001) indicate significant difference from the absence of mefloquine (ANOVA).

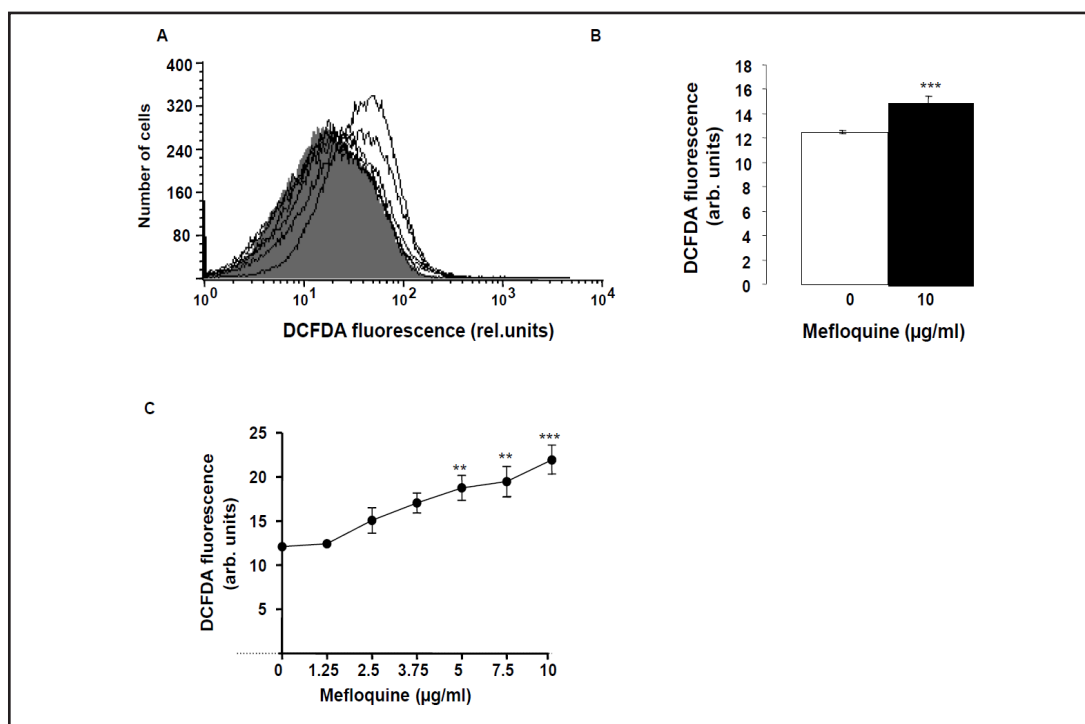


Fig. 3. Effect of mefloquine on reactive oxygen species. A. Original histogram of 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (grey shadow) and with (black lines) presence of 1.25–10 µg/ml mefloquine. B. Arithmetic means \pm SEM ($n = 4$) of the erythrocyte DCFDA fluorescence following incubation for 48 h to Ringer solution without (white bar) or with (black bar) presence of 10 µg/ml mefloquine. ***($P < 0.001$) indicates significant difference from the absence of mefloquine (t test). C. DCFDA fluorescence as a linear function of mefloquine concentration ($n = 9$). **($P < 0.01$), *** ($P < 0.001$) indicate significant difference from the absence of mefloquine (ANOVA).

was followed by an increase of DCFDA fluorescence, an effect reaching statistical significance at 5 µg/ml mefloquine concentration. Accordingly, mefloquine induces oxidative stress.

Oxidative stress may open Ca^{2+} permeable cation channels with subsequent Ca^{2+} entry. Additional experiments were thus performed exploring whether mefloquine influences cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Fluo3 fluorescence was employed to quantify $[\text{Ca}^{2+}]_i$. As shown in Fig. 4, a 48 h exposure to mefloquine (1.25–10 µg/ml) increased Fluo3 fluorescence reflecting increase of $[\text{Ca}^{2+}]_i$, an effect reaching statistical significance at 7.5 µg/ml mefloquine concentration. An additional series of experiments was performed in order to test whether mefloquine-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 10 µg/ml mefloquine, both in the presence or nominal absence of extracellular Ca^{2+} . As shown in Fig. 4E, removal of extracellular Ca^{2+} significantly blunted the effect of mefloquine on annexin-V-binding, an observation indicating that entry of extracellular Ca^{2+} contributed to the stimulation of cell membrane scrambling by mefloquine. However, even in the absence of extracellular Ca^{2+} , mefloquine significantly increased the percentage of annexin-V-binding erythrocytes, indicating that eryptosis was in part due to mechanisms other than entry of extracellular Ca^{2+} .

Mechanisms stimulating cell membrane scrambling even without Ca^{2+} entry and subsequent increase of $[\text{Ca}^{2+}]_i$ include ceramide. Accordingly, the ceramide abundance at the erythrocyte surface was determined utilizing a specific anti-ceramide antibody. As shown in Fig. 5, exposing erythrocytes for 48 hours to 1.25–10 µg/ml mefloquine was followed by an increase of the ceramide abundance at the erythrocyte surface, an effect reaching statistical significance at 10 µg/ml mefloquine concentration.

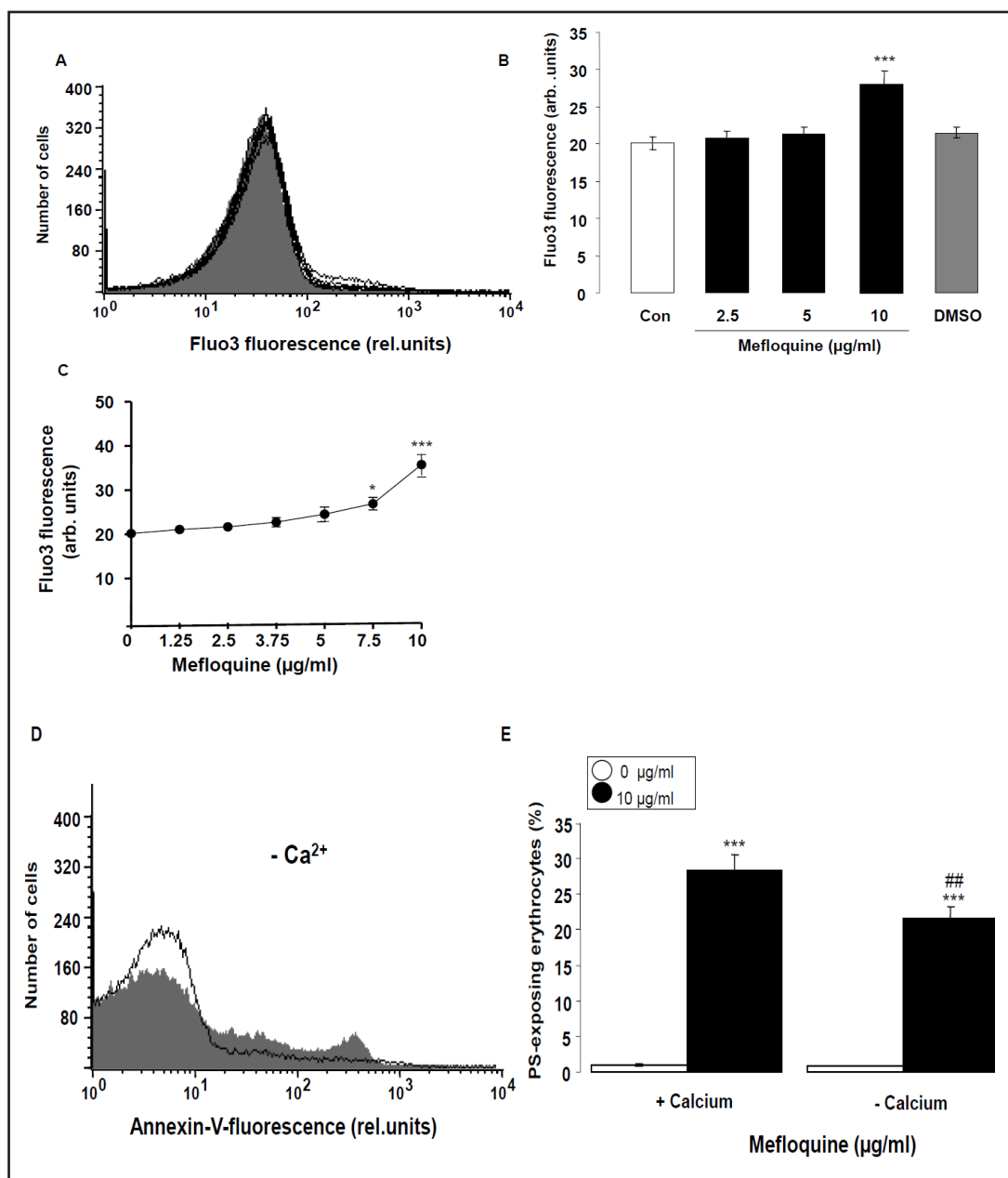


Fig. 4. Effect of mefloquine on erythrocyte Ca^{2+} activity and Ca^{2+} dependence of mefloquine-induced phosphatidylserine exposure. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black lines) presence of 1.25-10 $\mu\text{g/ml}$ mefloquine. B. Arithmetic means \pm SEM ($n = 10$) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) mefloquine (1.25 - 10 $\mu\text{g/ml}$). ***($P < 0.001$) indicates significant difference from the absence of mefloquine (ANOVA). C. Fluo3 fluorescence as a function of mefloquine concentration ($n = 15$). *($P < 0.05$), ***($P < 0.001$) indicate significant difference from the absence of mefloquine (ANOVA). D. Original histogram of annexin V binding of erythrocytes following exposure for 48 h to Ringer solution in the absence of calcium, without (grey area) and with (black line) presence of 10 $\mu\text{g/ml}$ mefloquine. E. Arithmetic means \pm SEM ($n = 8$) of erythrocyte annexin-V-binding after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 10 $\mu\text{g/ml}$ mefloquine in the presence (left bars, +Calcium) and absence (right bars, -Calcium) of Ca^{2+} . ***($P < 0.001$) indicates significant difference from the absence of mefloquine, ##($P < 0.01$) indicates significant difference from the respective value in the presence of Ca^{2+} .

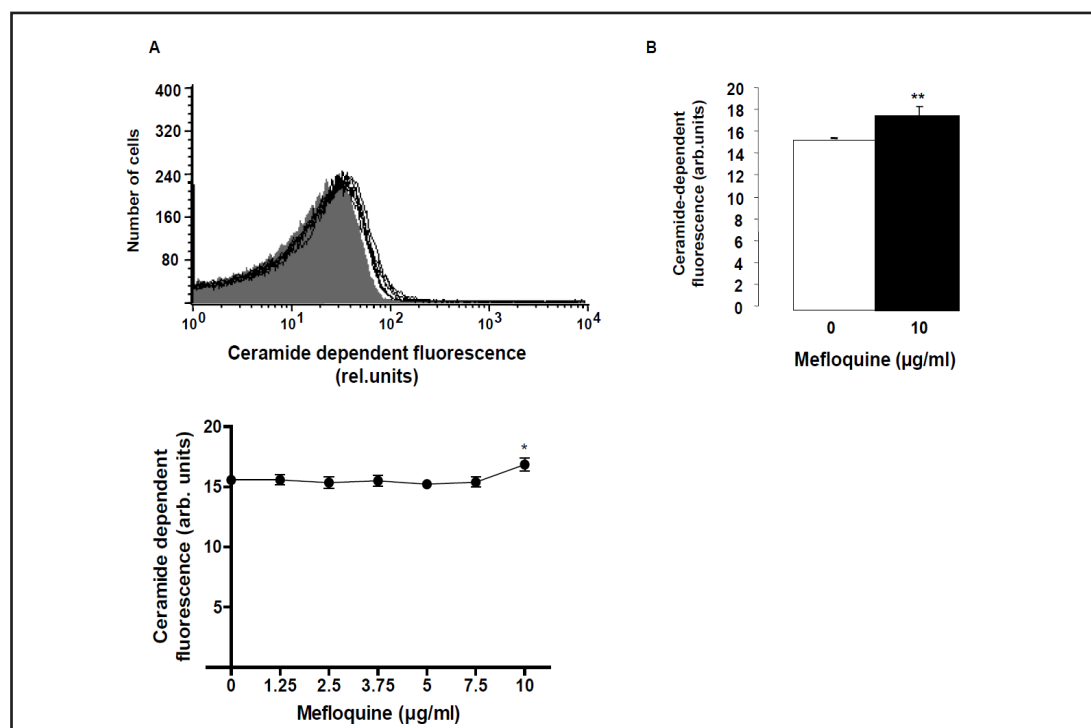


Fig. 5. Effect of mefloquine on ceramide abundance. A. Original histogram of ceramide abundance at the erythrocyte surface following exposure for 48 h to Ringer solution without (grey shadow) and with (black lines) presence of 1.25–10 µg/ml mefloquine. B. Arithmetic means \pm SEM (n= 4) of ceramide abundance after a 48 h incubation in Ringer solution without (white bar) or with 10 µg/ml mefloquine (black bar). ** ($P<0.01$) indicates significant difference from the absence of mefloquine (t test). C. Ceramide abundance as a function of mefloquine concentration (n=10). * ($P<0.05$) indicates significant difference from the absence of mefloquine (ANOVA).

Discussion

The present observations unravel a novel effect of mefloquine, i.e. the triggering of cell shrinkage and erythrocyte cell membrane scrambling with phosphatidylserine translocation from the cell interior to the erythrocyte surface. Cell shrinkage and cell membrane scrambling are the hallmarks of eryptosis, the suicidal erythrocyte death. The mefloquine concentration required for stimulation of erythrocyte cell membrane scrambling is similar (forward scatter) or slightly higher (annexin-V-binding) than the plasma concentrations (2.5 µg/ml) reported *in vivo* [66]. The observations are at variance to an earlier paper failing to observe an effect of mefloquine on phosphatidylserine abundance at the erythrocyte surface [67].

The mefloquine induced erythrocyte shrinkage was paralleled by and is at least partially due to increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), which activates Ca^{2+} sensitive K^+ channels leading to K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with osmotically obliged water [24].

The increase of $[\text{Ca}^{2+}]_i$ may have been due to oxidative stress, which activates oxidant sensitive Ca^{2+} permeable erythrocytic cation channels [25], thus contributing to mefloquine induced Ca^{2+} entry. Mefloquine has similarly been reported to induce oxidative stress in nucleated cells [10, 18, 21].

Removal of extracellular Ca^{2+} significantly blunted the stimulation of annexin-V-binding following mefloquine treatment, indicating that Ca^{2+} entry contributes to the stimulation of mefloquine induced cell membrane scrambling. However, mefloquine enhanced the phosphatidylserine abundance at the cell surface significantly even in the absence of extracellular Ca^{2+} . Thus, the effect of mefloquine on Ca^{2+} entry contributed to, but did not fully

account for the triggering of cell membrane scrambling. Accordingly, mefloquine triggered cell membrane scrambling through additional, Ca^{2+} independent mechanisms.

Mechanisms triggering cell membrane scrambling even at constant $[\text{Ca}^{2+}]_i$ include ceramide [25]. Mefloquine treatment indeed enhanced the ceramide abundance in the erythrocyte cell membrane. The increase could result from either, formation of new ceramide or translocation of preexisting ceramide to the erythrocyte surface. Mefloquine has previously been shown to increase ceramide formation in nucleated cells [22].

The physiological purpose of eryptosis is the disposal of defective erythrocytes and thus avoidance of hemolysis [25]. Hemolysis leads to release of hemoglobin, which may be filtered in the kidney and precipitate in the acidic lumen of renal tubules thus occluding affected nephrons [68]. Eryptosis may further lead to removal of infected erythrocytes during malaria [23]. The malaria pathogen *Plasmodium* imposes oxidative stress on the host erythrocyte, which in turn activates several host cell ion channels including Ca^{2+} -permeable erythrocyte cation channels [25, 69]. The Ca^{2+} entry through those channels eventually leads to cell membrane scrambling of the infected erythrocytes with phosphatidylserine dependent phagocytosis and thus clearance from circulating blood [23]. The removal of infected phosphatidylserine exposing erythrocytes lowers the parasitemia and thus favourably influences the clinical course of malaria. Along those lines, enhanced erythrocyte susceptibility to triggers of eryptosis may protect carriers of several genetic erythrocyte disorders, such as sickle-cell trait, beta-thalassemia-trait, homozygous Hb-C and homozygous G6PD-deficiency, against a severe course of malaria [25, 70-72]. Stimulated eryptosis may further contribute to the protective effect against malaria of iron deficiency [73], lead intoxication [73], treatment with chlorpromazine [74] or presence of NO synthase inhibitors [74]. It is tempting to speculate that the antimalarial effect of mefloquine could be in part due to stimulation of eryptosis. Needless to say that additional experimentation would be required to confirm or falsify this speculation.

In conclusion, mefloquine stimulates erythrocyte cell membrane scrambling, an effect paralleled by and at least partially resulting from induction of oxidative stress, increase of cytosolic Ca^{2+} activity and enhanced ceramide abundance at the erythrocyte surface.

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Disclosure Statement

No conflict of interest.

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