

Original Paper

Stimulating Effect of Terfenadine on Erythrocyte Cell Membrane Scrambling

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Key Words

Phosphatidylserine • Ionomycin • Eryptosis • Calcium • Terfenadine

Abstract

Background/Aims: The antihistaminic drug Terfenadine may trigger apoptosis of tumor cells, an effect unrelated to its effect on histamine receptors. Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal death of erythrocytes characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Signaling triggering eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), oxidative stress, and ceramide. The present study explored, whether Terfenadine is capable to trigger eryptosis. **Methods:** Flow cytometry was employed to estimate phosphatidylserine abundance at the erythrocyte surface from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, abundance of reactive oxygen species (ROS) from 2',7'-dichlorodihydrofluorescein (DCF) diacetate dependent fluorescence, and ceramide abundance at the human erythrocyte surface utilizing specific antibodies. Hemolysis was quantified from haemoglobin concentration in the supernatant. **Results:** A 48 hours exposure of human erythrocytes to Terfenadine ($\geq 5 \mu\text{M}$) significantly increased the percentage of annexin-V-binding cells and triggered hemolysis without significantly modifying the average forward scatter. Terfenadine ($7.5 \mu\text{M}$) significantly increased Fluo3-fluorescence, but did not significantly modify DCF fluorescence or ceramide abundance. The effect of Terfenadine on annexin-V-binding was significantly blunted but not abolished by removal of extracellular Ca^{2+} . Exposure of human erythrocytes to Ca^{2+} ionophore ionomycin ($1 \mu\text{M}$, 15 min) triggered annexin-V-binding, an effect augmented by Terfenadine pretreatment ($10 \mu\text{M}$, 48 hours). **Conclusions:** Terfenadine triggers phospholipid scrambling of the human erythrocyte cell membrane, an effect in part due to entry of extracellular Ca^{2+} and in part due to sensitizing human erythrocyte cell membrane scrambling to Ca^{2+} .

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Introduction

Terfenadine, a selective histamine H1 receptor antagonist [1-8], is clinically used against allergic disorders such as hay fever, allergic rhinitis, and other histamine-mediated disorders [6, 8-10]. The substance does not impair psychomotor performance to a similar extent as traditional antihistamines [4, 6-8]. Besides blocking the H1 receptor, Terfenadine may interfere with degranulation of mast cells and thus decrease histamine release [5]. Terfenadine and related substances may further trigger apoptosis [11-18] and may thus be effective against malignancy [12-15, 17, 19-21]. The effect is unrelated to antihistaminergic activity [11]. Instead, Terfenadine is in part effective by mitochondrial depolarization [16], cytochrome c release [16], oxidative stress [12], increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [11] and activation of caspases [12, 15, 16].

Similar to apoptosis of tumor cells erythrocytes may enter eryptosis, the suicidal death of erythrocytes characterized by cell shrinkage [22] and cell membrane scrambling with breakdown of phosphatidylserine asymmetry of the cell membrane and phosphatidylserine translocation to the cell surface [23]. Signaling involved in the stimulation of eryptosis include Ca^{2+} entry with increase of $[\text{Ca}^{2+}]_i$ [23], ceramide [24], oxidative stress [23], energy depletion [23], caspases [23, 25, 26], as well as dysfunction of several kinases, such as casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and p38 kinase, PAK2 kinase, AMP-activated kinase AMPK, cGMP-dependent protein kinase, and sorafenib/sunitinib sensitive kinases [23].

The present study explored, whether and how Terfenadine modifies eryptosis. To this end, human erythrocytes drawn from healthy volunteers were treated with Terfenadine and phosphatidylserine surface abundance, cell volume, hemolysis, $[\text{Ca}^{2+}]_i$, abundance of reactive oxygen species (ROS) and ceramide 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 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, 1 CaCl_2 , at 37°C for 48 hours. Where indicated, erythrocytes were exposed for 48 hours to Terfenadine (Sigma Aldrich, Hamburg, Germany) or for 15 min to Ca^{2+} ionophore ionomycin (Sigma Aldrich, Hamburg, Germany).

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 100 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 20 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 Terfenadine 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 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. 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 100 μ l suspension of erythrocytes was washed in Ringer solution and 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 washed two 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).

Ceramide abundance

For the determination of ceramide, a monoclonal antibody-based assay was used. To this end, cells were stained for 1 hour at 37°C with 1 μ g/ml anti ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. The samples were washed twice with PBS-BSA. The 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 analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. As a control, secondary antibody alone was used.

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 have not necessarily identical susceptibility to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study explored the effect of Terfenadine on suicidal erythrocyte death or eryptosis, which is characterized by cell shrinkage and by break-down of phospholipid asymmetry of the cell membrane with phosphatidylserine translocation to the cell surface.

Forward scatter was determined by flow cytometry as a measure of erythrocyte volume. The measurements were performed after incubation of the erythrocytes 48 hours in Ringer solution without or with Terfenadine (1 – 10 μ M). As shown in Fig. 1, Terfenadine did not significantly modify the average erythrocyte forward scatter; Terfenadine tended to decrease the percentage both of swollen and of shrunken erythrocytes, an effect, however, not reaching statistical significance (Fig. 1C, D).

Annexin binding determined by flow cytometry was taken as a measure of cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with Terfenadine (1 – 10 μ M). As shown in Fig. 2, a 48 hours exposure to Terfenadine increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 5 μ M Terfenadine.

Hemoglobin in the supernatant was determined to quantify the percentage of hemolytic erythrocytes. As illustrated in Fig. 3, a 48 hours exposure to Terfenadine increased the percentage of hemolytic erythrocytes, an effect reaching statistical significance at 5 μ M Terfenadine concentration. Thus, Terfenadine triggered hemolysis.

Fluo3-fluorescence was employed to measure cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). As illustrated in Fig. 4, a 48 hours exposure to Terfenadine increased the Fluo3-fluorescence, an effect reaching statistical significance at 7.5 μ M Terfenadine concentration. Thus, Terfenadine increased $[\text{Ca}^{2+}]_i$.

In order to test whether the Terfenadine-induced translocation of phosphatidylserine was sensitive to extracellular Ca^{2+} , erythrocytes were incubated for 48 hours in the absence

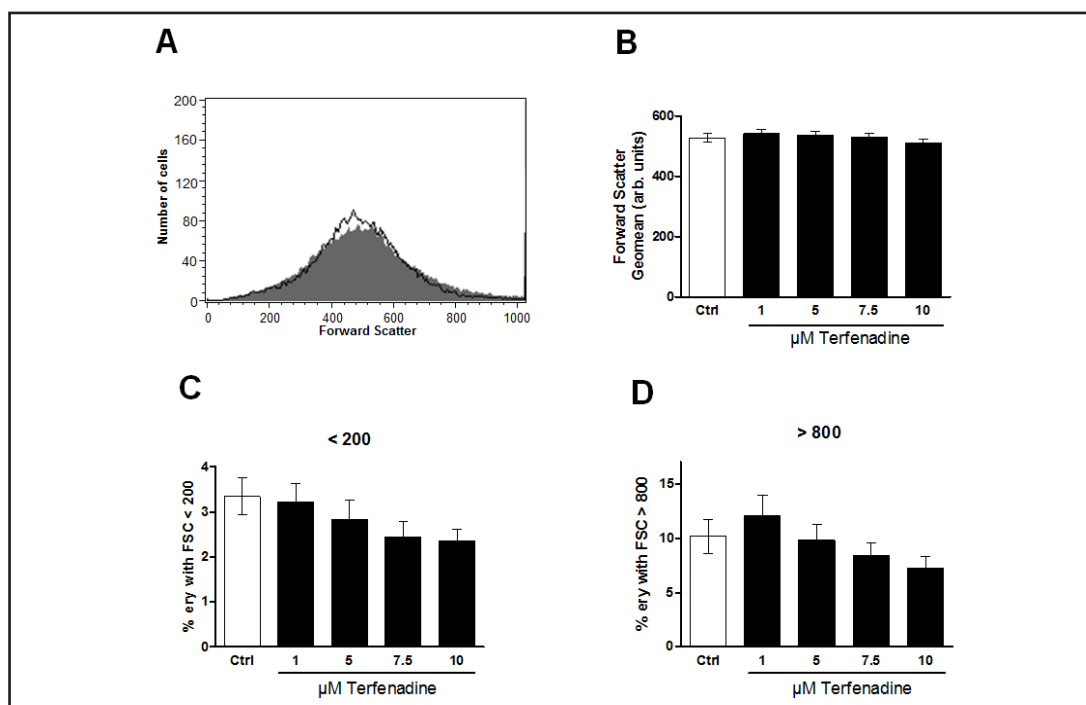


Fig. 1. Effect of Terfenadine on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 10 μ M Terfenadine. B. Arithmetic means \pm SEM ($n = 14$) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Terfenadine (1 - 10 μ M). C. Arithmetic means \pm SEM ($n = 14$) of the percentage erythrocytes with forward scatter (FSC) <200 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Terfenadine (1 - 10 μ M). D. Arithmetic means \pm SEM ($n = 14$) of the percentage erythrocytes with forward scatter (FSC) >800 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Terfenadine (1 - 10 μ M).

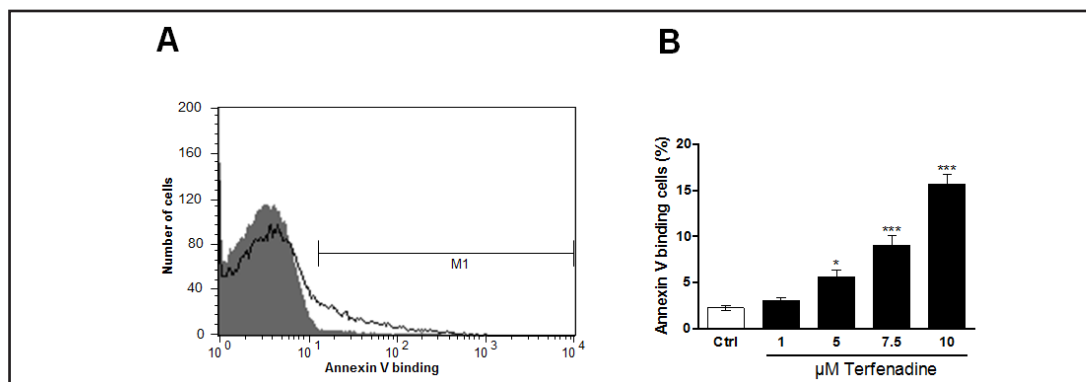


Fig. 2. Effect of Terfenadine on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 10 μ M Terfenadine. B. Arithmetic means \pm SEM ($n = 14$) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Terfenadine (1 - 10 μ M). *($p < 0.05$), ***($p < 0.001$) indicates significant difference from the absence of Terfenadine (ANOVA).

or presence of 10 μ M Terfenadine in the presence or nominal absence of extracellular Ca^{2+} . As shown in Fig. 5, removal of extracellular Ca^{2+} significantly blunted the effect of Terfenadine on the percentage of annexin-V-binding erythrocytes. However, even in the absence of

Fig. 3. Effect of Terfenadine on hemolysis. Arithmetic means \pm SEM ($n = 14$) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Terfenadine (1 - 10 μ M). ***($p < 0.001$) indicates significant difference from the absence of Terfenadine (ANOVA).

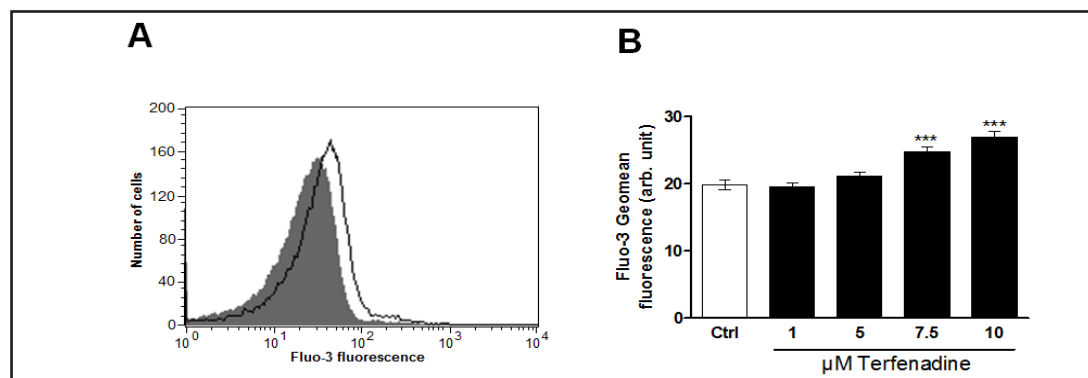
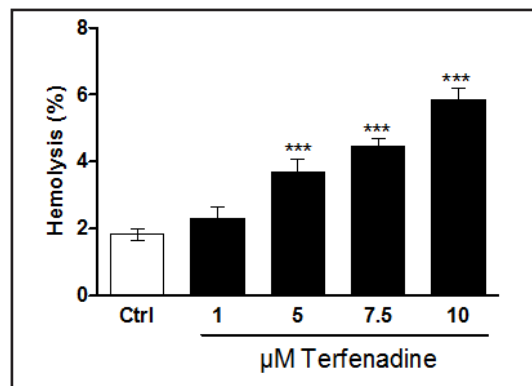


Fig. 4. Effect of Terfenadine on cytosolic Ca^{2+} activity. A. Original histogram of Fluo3-fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 10 μ M Terfenadine. B. Arithmetic means \pm SEM ($n = 14$) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Terfenadine (1 - 10 μ M). ***($p < 0.001$) indicates significant difference from the absence of Terfenadine (ANOVA).

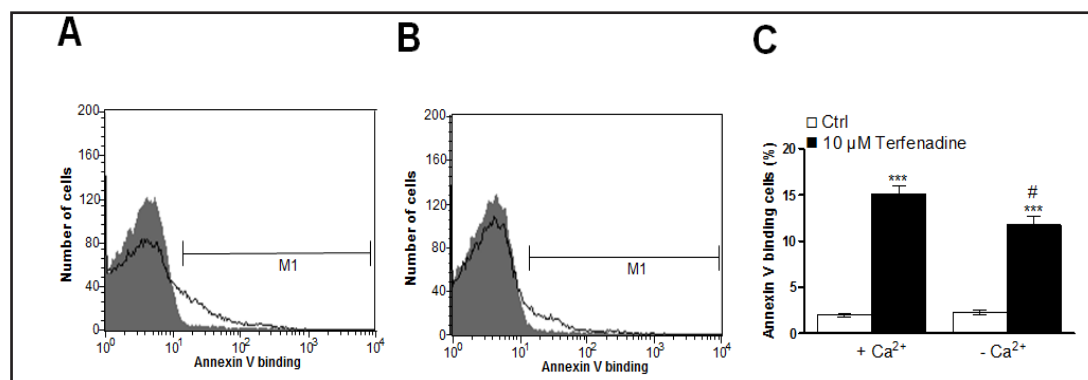


Fig. 5. Ca^{2+} sensitivity of Terfenadine-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) Terfenadine (10 μ M) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means \pm SEM ($n = 16$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) Terfenadine (10 μ M) in the presence (left bars, + Ca^{2+}) and absence (right bars, - Ca^{2+}) of Ca^{2+} . ***($p < 0.001$) indicates significant difference from the absence of Terfenadine, #($p < 0.05$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

extracellular Ca^{2+} , Terfenadine significantly increased the percentage of annexin-V-binding erythrocytes. Thus, Terfenadine was effective in part, but not fully by stimulating entry of extracellular Ca^{2+} .

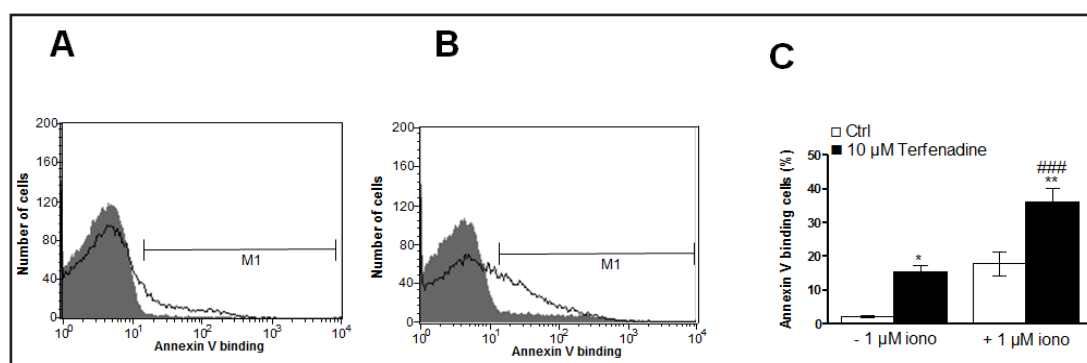


Fig. 6. Terfenadine-sensitivity of ionomycin-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes without (grey area) and with (black line) a 48 hours Terfenadine (10 μM) pretreatment and without (A) and with (B) a 15 min exposure to ionomycin (1 μM). C. Arithmetic means \pm SEM (n = 5) of annexin-V-binding of erythrocytes without (left bars, - 1 μM iono) and with (right bars, + 1 μM iono) a 15 min exposure to ionomycin (1 μM) without (white bars) and with (black bars) a 48 hours Terfenadine (10 μM) pretreatment. *(p<0.05), ***(p<0.001) indicates significant difference from the absence of Terfenadine, ###(p<0.001) indicates significant difference from the absence of ionomycin (ANOVA).

In order to test whether Terfenadine enhanced the Ca^{2+} sensitivity of cell membrane scrambling, erythrocytes were incubated for 48 hours in the absence or presence of 10 μM Terfenadine and subsequently loaded with Ca^{2+} by a 15 minute treatment with the Ca^{2+} ionophore ionomycin (1 μM). As shown in Fig. 6, the pretreatment with 10 μM Terfenadine significantly increased the percentage of annexin-V-binding erythrocytes and significantly augmented the stimulating effect of ionomycin on the percentage of annexin-V-binding erythrocytes. Thus, Terfenadine sensitized the erythrocytes for the scrambling effect of Ca^{2+} entry.

In order to test, whether Terfenadine induces oxidative stress, the abundance of reactive oxygen species (ROS) was quantified utilizing 2',7'-dichlorodihydrofluorescein (DCF) diacetate. As a result, the DCF fluorescence was similar following a 48 hours incubation in Ringer with 10 μM Terfenadine (22.2 ± 4.2 a.u., n = 10) and without Terfenadine (24.7 ± 3.2 a.u., n = 10). Thus, Terfenadine did not appreciably induce oxidative stress.

In order to quantify ceramide abundance at the erythrocyte surface specific antibodies were used. As a result, the ceramide abundance was similar following a 48 hours incubation in Ringer with 10 μM Terfenadine (9.9 ± 0.3 a.u., n = 5) and in the absence of Terfenadine (11.3 ± 0.4 a.u., n = 5). Thus, Terfenadine did not appreciably induce ceramide abundance.

Discussion

The present observations uncover a novel effect of Terfenadine, i.e. the stimulation of erythrocyte cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentrations required for this effect are in the range of the concentrations required to trigger apoptosis of tumor cells [11]. Terfenadine may be particularly effective in clinical conditions with accelerated eryptosis, such as dehydration [27], hyperphosphatemia [28], chronic kidney disease (CKD) [29-32], hemolytic-uremic syndrome [33], diabetes [34], hepatic failure [35], malignancy [23], sepsis [36], sickle-cell disease [23], beta-thalassemia [23], Hb-C and G6PD-deficiency [23], as well as Wilsons disease [37]. Moreover, Terfenadine may augment the eryptotic effect of other xenobiotics [23, 38-66] possibly leading to serious drug-drug interactions [67, 68].

The effect of Terfenadine on cell membrane scrambling was paralleled by an increase of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and blunted by removal of Ca^{2+} from extracellular space, indicating that entry of extracellular Ca^{2+} contributed to the stimulating effect of Terfenadine

on phosphatidylserine translocation. Terfenadine did not appreciably influence the average forward scatter even though an increase of $[Ca^{2+}]_i$ were expected to trigger activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [22]. Thus, some other effect of Terfenadine apparently prevented the decrease of cell volume despite increase of $[Ca^{2+}]_i$.

Moreover, Terfenadine triggered cell membrane scrambling even in the absence of extracellular Ca^{2+} , indicating that Terfenadine triggered cell membrane scrambling in part by mechanisms other than Ca^{2+} entry. Terfenadine further augmented the cell membrane scrambling triggered by ionomycin, a Ca^{2+} ionophore flooding the cell with Ca^{2+} . Thus, Terfenadine sensitizes apparently the cell membrane scrambling to the stimulating effects of Ca^{2+} . Additional mechanisms able to trigger eryptosis include oxidative stress and ceramide [23]. However, Terfenadine triggered cell membrane scrambling without enhancing the abundance of reactive oxygen species or ceramide. Thus, the additional mechanism(s) involved in the stimulation of erythrocyte cell membrane scrambling remained elusive.

Besides inducing cell membrane scrambling, Terfenadine triggers hemolysis. Hemolysis leads to release of hemoglobin, which passes the renal glomerular filter, precipitates in the acidic lumen of renal tubules, occludes nephrons and thus may lead to renal failure [69]. As a matter of fact eryptosis is intended to precede hemolysis and thus to prevent the release of hemoglobin [23]. Eryptosis further counteracts development of parasitemia in malaria, as infected erythrocytes enter eryptosis and are thus cleared from circulating blood [23].

If the loss of erythrocytes due to stimulation of eryptosis and subsequent clearance of phosphatidylserine exposing erythrocytes from circulating blood outcasts the formation of new erythrocytes by erythropoiesis, anemia develops [23]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [70], stimulate blood clotting and thus foster thrombosis [71-73]. Stimulation of eryptosis may thus impair microcirculation [24, 71, 74-77].

In conclusion, Terfenadine triggers cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface, an effect in part due to Ca^{2+} entry.

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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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