Original Paper

Camalexin-Induced Cell Membrane Scrambling and Cell Shrinkage in Human Erythrocytes

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Key Words

Phosphatidylserine • Cell volume • Eryptosis • Calcium • Staurosporine • Chelerythrine • zVAD

Abstract

Background/Aims: The thaliana phytoalexin Camalexin has been proposed for the treatment of malignancy. Camalexin counteracts tumor growth in part by stimulation of suicidal death or apoptosis of tumor cells. Similar to apoptosis of nucleated cells, erythrocytes may enter suicidal death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Cellular mechanisms contributing to the complex machinery executing eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), oxidative stress, ceramide, protein kinase C and caspases. The present study explored, whether Camalexin induces eryptosis and, if so, to shed light on mechanisms involved. **Methods:** Phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo-3 fluorescence, ROS formation from DCFDA dependent fluorescence, and ceramide abundance utilizing specific antibodies. **Results:** A 48 hours exposure of human erythrocytes to Camalexin significantly increased the percentage of annexin-V-binding cells ($\geq 10 \mu\text{g/ml}$), significantly decreased forward scatter ($\geq 5 \mu\text{g/ml}$) and significantly increased Fluo-3-fluorescence ($\geq 10 \mu\text{g/ml}$), but did not significantly modify DCFDA fluorescence or ceramide abundance. The effect of Camalexin on annexin-V-binding was significantly blunted by removal of extracellular Ca^{2+} , by kinase inhibitors staurosporine ($1 \mu\text{M}$) and chelerythrine ($10 \mu\text{M}$), as well as by caspase inhibitors zVAD ($10 \mu\text{M}$) and zIETD-fmk ($50 \mu\text{M}$). **Conclusions:** Camalexin triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect at least in part depending on Ca^{2+} entry, as well as staurosporine and chelerythrine sensitive kinase(s) as well as zVAD and zIETD-fmk sensitive caspase(s).

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Introduction

The thaliana phytoalexin and indole alkaloid camalexin (3-thiazol-2'-yl-indole) [1-4] participates in the defense of plants against pathogens [2, 5-8]. Moreover, the substance is effective against cancer cells [2, 9]. Camalexin counteracts malignancy at least in part by triggering of cancer cell apoptosis [10-13].

In analogy to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal death of erythrocytes [14]. Hallmarks of eryptosis are cell shrinkage [15] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [14], events in common with apoptosis of nucleated cells. Erythrocytes lack, however nuclei and mitochondria, key organelles of apoptosis. Nevertheless apoptosis and eryptosis have a wide variety of triggers and signalling pathways in common [14]. Signaling involved in the triggering of eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [14], ceramide [16], and oxidative stress [14]. Some stimulators of eryptosis are effective by activation of caspases [14, 17, 18]. Triggering of eryptosis may further involve stimulation of kinases [14]. Eryptosis is triggered by multiple xenobiotics [14, 19-65]. Moreover, enhanced eryptosis is observed in several clinical conditions including dehydration [14], hyperphosphatemia [14], chronic kidney disease (CKD) [66, 67], hemolytic-uremic syndrome [14], diabetes [68], hepatic failure [69], malignancy [14], sepsis [70], sickle-cell disease [14], beta-thalassemia [14], Hb-C and G6PD-deficiency [14], as well as Wilson's disease [71].

The present study explored, whether Camalexin modifies eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to Camalexin and phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$, ROS formation, 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 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 5, 10, 15 and 20 $\mu\text{g}/\text{ml}$ Camalexin (MedChem Express, Princeton, USA). Given the molecular weight of Camalexin (200 g/mol), 5 $\mu\text{g}/\text{ml}$ corresponds to 25 μM . In order to estimate the impact of Ca^{2+} entry, erythrocytes were exposed to Camalexin in the absence of extracellular Ca^{2+} and presence of Ca^{2+} chelator EGTA (1 mM, Merck Millipore, Darmstadt, Germany). To test for an involvement of kinases, erythrocytes were exposed for 48 hours to a combination of Camalexin and protein kinase C inhibitor staurosporine (Sigma Aldrich, Hamburg, Germany), or p38 kinase inhibitor SB 203580 (Tocris bioscience, Bristol, UK), or casein kinase inhibitor D4476 (Sigma Aldrich, Hamburg, Germany). In order to test for a role of caspases, erythrocytes were exposed for 48 hours to a combination of Camalexin and pan-caspase inhibitor zVAD (Tocris bioscience, Bristol, UK),

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 high CaCl_2 concentration is required for the binding of annexin-V to phosphatidylserine. 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 Camalexin 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 Ca²⁺

After incubation, erythrocytes were washed in Ringer solution and 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. Ca²⁺-dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Reactive oxidant species (ROS)

Oxidative stress was determined utilizing 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 μl suspension of erythrocytes was washed in Ringer solution and stained with DCFDA (Sigma, Schnellendorf, 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 washed two times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 μl Ringer solution and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

Ceramide abundance

For the determination of ceramide, a monoclonal antibody-based assay was used. To this end, cells were stained for 1 hour at 37°C with 1 μg/ml anti-ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. The samples were washed twice with PBS-BSA. 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.

Hemolysis

Following incubation, the erythrocyte suspension was centrifuged for 3 min at 1600 rpm, 4°C, and the supernatant harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration in the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

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 Camalexin stimulates eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Erythrocyte volume was estimated from forward scatter which was determined utilizing flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with Camalexin (5, 10, 15 and 20 μg/ml). As illustrated in Fig. 1, Camalexin decreased erythrocyte forward scatter, an effect reaching statistical significance at 5 μg/ml Camalexin concentration.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. Prior to measurements, the erythrocytes were again

Fig. 3. Effect of Camalexin on cytosolic Ca²⁺ concentration. A. Original histogram of Fluo-3 fluorescence reflecting cytosolic Ca²⁺ concentration in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 20 µg/ml Camalexin. B. Arithmetic means ± SEM (n = 10) of Fluo-3 fluorescence in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Camalexin (5, 10, 15 and 20 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). *** (p<0.001) indicates significant difference from the absence of Camalexin (ANOVA).

incubated for 48 hours in Ringer solution without or with Camalexin (5, 10, 15 and 20 µg/ml). As shown in Fig. 2, a 48 hours exposure to Camalexin increased the percentage of

Fig. 4. Ca^{2+} sensitivity of Camalexin-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) Camalexin (20 $\mu\text{g}/\text{ml}$) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means $\pm$ SEM ($n = 10$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) Camalexin (20 $\mu\text{g}/\text{ml}$) in the presence (+ Ca^{2+}) and absence (right bars, - Ca^{2+}) of Ca^{2+} . ***($p < 0.001$) indicates significant difference from the absence of Camalexin, ###($p < 0.001$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

Fig. 5. Staurosporine and chelerythrine sensitivity of Camalexin-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) Camalexin (20 $\mu\text{g}/\text{ml}$) in the absence (A) and presence of (B) 1 μM staurosporine or (C) 10 μM chelerythrine. Arithmetic means $\pm$ SEM ($n = 8$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) Camalexin (20 $\mu\text{g}/\text{ml}$) in the absence (left bars, + DMSO) and presence of 1 μM staurosporine (middle bars, + Staurosporine), or of 10 μM chelerythrine (right bars, + chelerythrine). *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$) indicates significant difference from the absence of Camalexin, #($p < 0.05$), ##($p < 0.01$) indicates significant difference from the absence of kinase inhibitors (ANOVA).

phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 10 $\mu\text{g}/\text{ml}$ Camalexin.

In order to test whether Camalexin triggers hemolysis, hemoglobin was determined in the supernatant. As a result, the percentage of hemolytic cells was similar following exposure

Fig. 6. zVAD sensitivity of Camalexin-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) Camalexin (20 μg/ml) in the (A) absence and presence of (B) 10 μM pan-caspase inhibitor zVAD or of (C) 50 μM caspase 8 inhibitor zIETD-fmk. Arithmetic means ± SEM (n = 10) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) Camalexin (20 μg/ml) in the absence (left bars, DMSO) and presence of 10 μM zVAD (middle bars, + zVAD) or of 50 μM zIETD-fmk (right bars, + zIETD-fmk). *(p<0.05), *** (p<0.001) indicates significant difference from the absence of Camalexin, ## (p<0.01) indicates significant difference from the absence of caspase inhibitors (ANOVA).

to 20 μg/ml Camalexin ($5.1 \pm 0.8\%$, n = 4) and in the absence of Camalexin ($3.5 \pm 0.8\%$, n = 4). Thus, Camalexin did not significantly increase hemolysis.

Fluo3 fluorescence was taken as a measure of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with Camalexin (5, 10, 15 and 20 μg/ml). As shown in Fig. 3, a 48 hours exposure to Camalexin increased the Fluo-3 fluorescence, an effect reaching statistical significance at 10 μg/ml Camalexin.

A next series of experiments explored whether the Camalexin-induced translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular Ca^{2+} . To this end, erythrocytes were incubated for 48 hours in the absence or presence of 20 μg/ml Camalexin in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 4, removal of extracellular Ca^{2+} significantly blunted the effect of Camalexin on the percentage of annexin-V-binding erythrocytes. Thus, Camalexin-induced cell membrane scrambling was at least in part triggered by entry of extracellular Ca^{2+} .

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As a result, the DCFDA fluorescence was similar following exposure to 20 μg/ml Camalexin (16.3 ± 2.0 , a.u., n = 10) and in the absence of Camalexin (15.3 ± 1.6 , a.u., n = 10). Thus, Camalexin did not appreciably induce oxidative stress (data not shown).

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As a result, the ceramide abundance was similar following exposure to 20 μg/ml Camalexin (7.5 ± 0.5 a.u., n = 10) and in the absence of Camalexin (7.1 ± 0.8 a.u., n = 10). Thus, Camalexin did not appreciably induce ceramide abundance (data not shown).

To explore, whether the effects of Camalexin involved kinase activity, the influence of Camalexin on annexin-V-binding and forward scatter was tested in the presence of protein kinase C inhibitors staurosporine (1 μ M) and chelerythrine (10 μ M), p38 kinase inhibitor SB 203580 (2 μ M) or casein kinase inhibitor D4476 (10 μ M). As illustrated in Fig. 5, the effect of Camalexin (20 μ g/ml) on phosphatidylserine exposure was significantly blunted by staurosporine and chelerythrine. The percentage of annexin-V-binding erythrocytes following exposure to 20 μ g/ml Camalexin was, however, similar in the absence of inhibitors (15.7 $\pm$ 3.0 a.u., n = 10) and in the presence of either SB203580 (11.3 $\pm$ 6.8 a.u., n = 10, data not shown) or D4476 (12.4 $\pm$ 4.5 a.u., n = 10, data not shown). Thus, the full effect of Camalexin required staurosporine and chelerythrine sensitive but not SB203580 sensitive or D4476 sensitive kinases.

In order to test for the involvement of caspases, the influence of Camalexin on annexin-V-binding and forward scatter was tested in the presence of pancaspase inhibitor zVAD (10 μ M), of caspase-8 inhibitor zIETD-fmk (50 μ M), and of caspase-3 inhibitor zDEVD (50 μ M). As illustrated in Fig. 6, the effect of Camalexin (20 μ g/ml) on phosphatidylserine exposure was significantly blunted by zVAD and zIETD-fmk. The percentage of annexin-V-binding erythrocytes following exposure to 20 μ g/ml Camalexin was, however, similar in the absence of inhibitors (16.9 $\pm$ 2.9 a.u., n = 8) and in the presence of zDEVD-fmk (19.1 $\pm$ 1.9 a.u., n = 8, data not shown).

Discussion

The present observations reveal a stimulating effect of Camalexin on suicidal erythrocyte death or eryptosis. Exposure of human erythrocytes to Camalexin is followed by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. In contrast, at the concentrations tested, Camalexin did not significantly modify hemolysis.

The effect of Camalexin on eryptosis was paralleled by an increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Moreover, the effect of Camalexin on cell membrane scrambling was in large part dependent on Ca^{2+} entry from the extracellular space, as removal of extracellular Ca^{2+} significantly blunted the effect of Camalexin on phosphatidylserine translocation. Ca^{2+} entry from the extracellular space could further account for the Camalexin-induced cell shrinkage which could result from increase of $[\text{Ca}^{2+}]_i$ with subsequent activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water.

The effect of Camalexin on phosphatidylserine translocation further apparently involves staurosporine and chelerythrine sensitive kinases such as protein kinase C. Accordingly, the effect of Camalexin on cell membrane scrambling was significantly blunted in the presence of protein kinase C inhibitor staurosporine, but not in the presence of p38 kinase inhibitor SB203580 and casein kinase inhibitor D4476. Camalexin-induced cell membrane scrambling was further significantly blunted in the presence of pan-caspase inhibitor zVAD and caspase 8 inhibitor zIETD-fmk, thus apparently involving activation of caspases. Increase of cytosolic Ca^{2+} concentration, activity of staurosporine and chelerythrine sensitive kinases as well as activated caspases are well known triggers of eryptosis [14]. Camalexin triggered cell membrane scrambling and cell shrinkage without enhancing the ceramide abundance. Moreover, Camalexin triggered eryptosis without inducing oxidative stress. Both, ceramide and oxidative stress are well known stimulators of eryptosis [14].

Consequences of eryptosis include rapid clearance of defective erythrocytes from circulating blood [14]. Failure of erythrocyte injury to trigger eryptosis may lead to hemolysis with 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 [72]. The rapid clearance of phosphatidylserine exposing erythrocytes from circulating blood could result in anemia as soon as the loss of erythrocytes surpasses the formation

of new erythrocytes by erythropoiesis [14]. Phosphatidylserine exposing erythrocytes may further impair microcirculation [16, 73-77] due to adherence of eryptotic erythrocytes to the vascular wall [78], stimulation of blood clotting and triggering of thrombosis [73, 79, 80],

To the extent that the effective concentrations are reached in plasma of Camalexin treated patients, side effects of the drug could include anemia and impaired microcirculation with increased risk of thrombosis.

In conclusion, Camalexin triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect in large part dependent on Ca^{2+} entry, staurosporine sensitive kinases and zVAD-sensitive caspases.

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Disclosure Statement

The authors declare no conflict of interests.

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