Original Paper

Stimulation of Erythrocyte Cell Membrane Scrambling by Gedunin

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Key Words

Phosphatidylserine • Gedunin • Calcium • Malaria • Anemia • Cell volume • Eryptosis

Abstract

Background/Aims: Gedunin, an inhibitor of heat shock protein HSP90, triggers apoptosis of tumor cells and is thus effective against malignancy. Moreover, the drug has antimalarial potency. In analogy to apoptosis of nucleated cells, erythrocytes may enter suicidal death or eryptosis, which is characterized by cell shrinkage and by phosphatidylserine translocation to the erythrocyte surface. Eryptosis may be triggered by increase of cytosolic Ca^{2+} -activity ($[\text{Ca}^{2+}]_i$). The present study explored whether gedunin stimulates eryptosis. **Methods:** Forward scatter was determined to estimate cell volume, annexin V binding to identify phosphatidylserine-exposing erythrocytes, hemoglobin release to depict hemolysis, and Fluo3-fluorescence to quantify $[\text{Ca}^{2+}]_i$. **Results:** A 48 h exposure of human erythrocytes to gedunin significantly increased $[\text{Ca}^{2+}]_i$ (12 μM), significantly decreased forward scatter (24 μM) and significantly increased annexin-V-binding (12 μM). The effect of gedunin (24 μM) on annexin-V-binding was virtually abrogated by removal of extracellular Ca^{2+} . **Conclusion:** Gedunin stimulates suicidal erythrocyte death or eryptosis, an effect mainly if not exclusively due to stimulation of Ca^{2+} entry.

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Introduction

Gedunin ((1S,3aS,4aR,4bS,5R,6aR,10aR,10bR,12aS)-5-(Acetyloxy)-1-(3-furanyl)-1,5,6,6a,7,10a,10b,11,12,12a,decahydro-4b,7,7,10a,12a,-pentamethyloxireno[c]phenanthro[1,2-d]pyran-3,8 (3aH,4bH)-dione, Fig.1), a component of *oleum azadirachti* from dried seeds

of *Azadirachta indica* A. Juss. (family: *Meliaceae*) [1], has been shown to inhibit heat shock protein HSP90 thus triggering apoptosis of tumor cells [2-6]. The substance has thus been considered for the treatment of malignancy [3, 4, 6, 7]. Mechanisms invoked in the triggering of tumor cell apoptosis following gedunin treatment include inhibition of the antiapoptotic protein HSP90 and downregulation of the anti-apoptotic proteins HSP70 and HSP27 [3], activation of the caspases 3, 8, and 9 [8] and inhibition of sphingomyelin biosynthesis [9]. Gedunin has further been shown to be effective *in vitro* against *Plasmodium falciparum* [10-14]. The target accounting for the effects of gedunin on parasites remained illdefined.

In analogy to apoptosis of nucleated cells, erythrocytes may enter suicidal death or eryptosis, which is characterized by cell membrane scrambling and cell shrinkage [15]. Triggers of eryptosis include increase of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), e.g. following Ca^{2+} entry through Ca^{2+} -permeable cation channels [15]. Increased $[\text{Ca}^{2+}]_i$ activates Ca^{2+} -sensitive K^+ channels leading to cell shrinkage due to K^+ exit, hyperpolarization, Cl^- exit and thus cellular loss of KCl and osmotically obliged water [16]. Increased $[\text{Ca}^{2+}]_i$ further triggers phospholipid scrambling of the cell membrane with translocation of phosphatidylserine to the erythrocyte surface [15]. Signaling in the regulation of eryptosis includes in addition ceramide formation [15], caspase activation [17-21] and deranged regulation of several kinases such as AMP activated kinase AMPK [22], casein kinase 1 α [23, 24], cGMP-dependent protein kinase [25], Janus-activated kinase JAK3 [26], protein kinase C [27], p38 kinase [28], PAK2 kinase [29] as well as sorafenib [30] and sunitinib [31] sensitive kinases.

Eryptosis may be stimulated by a wide variety of xenobiotics [15, 31-66] and excessive eryptosis is further observed in several clinical conditions including diabetes, renal insufficiency, hemolytic uremic syndrome, sepsis, malaria, sickle cell disease, Wilson's disease, iron deficiency, malignancy, phosphate depletion, and metabolic syndrome [15].

Erythrocytes have previously been shown to express HSP90 [67, 68] and eryptosis could be stimulated by the HSP90 inhibitor geldanamycin [60].

The present study thus explored, whether and how gedunin influences eryptosis. To this end, $[\text{Ca}^{2+}]_i$, cell volume and phosphatidylserine translocation to the erythrocyte surface were determined in the absence and presence of gedunin.

Materials and Methods

Erythrocytes, solutions and chemicals

Erythrocytes were kindly provided by the blood bank of the University of Tübingen. For retrieval of leukocyte-free erythrocyte concentrates, citrate was used as anticoagulant. Prior to experiments, the concentrates were stored at 4°C in the commonly-used SAG mannitol solution (0.41 – 0.26 ml/ml concentrate) with CPD stabilisator solution (0.015 – 0.007 ml/ml concentrate). 100 ml SAG mannitol solution contained 0.877 g NaCl, 0.9 g glucose, 0.0169 g adenosine, 0.525 g mannitol. Hundred ml CPD stabilisator solution contained 0.327 g citric acid monohydrate, 2.63 g sodium citrate, 2.55 g glucose monohydrate, 0.251 g sodium dihydrogenphosphate. The erythrocyte concentrates were 7 – 20 days old when starting the experiment. The study is approved by the ethics committee of the University of Tübingen (184/2003V). 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), 5 glucose, 1 CaCl_2 , pH 7.4 at 37°C for 48 h. Where indicated, erythrocytes were exposed to gedunin (Tocris, Bristol, U.K.) at the indicated concentrations. In Ca^{2+} -free Ringer solution, 1 mM CaCl_2 was substituted by 1 mM glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). Gedunin was added from a stock solution (10 mM) dissolved in DMSO. The maximal final concentration of solvent was 2.4 μl .

FACS analysis of annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 50 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. In the following, the forward scatter (FSC) of the cells was determined, and 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). Alterations of forward scatter are paralleled by similar alterations of mean erythrocyte volume (MCV) [69]. No attempt was made to calculate mean erythrocyte cell volume from forward scatter.

Measurement of intracellular Ca^{2+}

After incubation erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl_2 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 CaCl_2 . The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl 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. As shown in previous papers [70], alterations of Ca^{2+} uptake leading to respective alterations of Fluo3 fluorescence are paralleled by similar alterations of radioactive Ca^{2+} fluxes. Alterations of Fluo3 fluorescence thus reflect alterations of cytosolic Ca^{2+} activity. However, the dye does not allow ratiometric measurements and thus does not allow calculating cytosolic Ca^{2+} activity.

Determination of ceramide formation

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 hour at 37°C with 1 $\mu\text{g}/\text{ml}$ 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. Subsequently, 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 then analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

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 putative effect of gedunin on eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling, both events stimulated by increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$).

In a first series of experiments, forward scatter was determined in flow cytometry as a measure of cell volume. Prior to measurements, erythrocytes were incubated in Ringer solution without or with gedunin (2–24 μM). As shown in Fig. 2, a 48 h exposure to gedunin was followed by a slight decrease of forward scatter, an effect reaching statistical significance at 24 μM gedunin concentration. Thus, exposure of erythrocytes to gedunin was followed by erythrocyte shrinkage.

The decrease of forward scatter could have resulted from activation of Ca^{2+} -sensitive K^+ channels with subsequent cell shrinkage due to exit of KCl and osmotically obliged water. Thus, the effect of gedunin on $[\text{Ca}^{2+}]_i$ was tested. Human erythrocytes were loaded with

Fig. 1. Structure of gedunin.

Fig. 2. Effect of gedunin 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 line) presence of 24 µM gedunin. B. Arithmetic means $\pm$ SEM ($n = 8$) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) gedunin (2–24 µM). * ($p < 0.05$) indicates significant difference from the absence of gedunin (ANOVA).

Fig. 3. Effect of gedunin on erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 24 µM gedunin. B,C. Original dot blots of annexin V binding as a function of forward scatter following exposure for 48 h to Ringer solution without (B) and with (C) presence of 24 µM gedunin. D. Arithmetic means $\pm$ SEM ($n = 8$) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) gedunin (2–24 µM). * ($p < 0.05$), *** ($p < 0.001$) indicate significant difference from the absence of gedunin (ANOVA).

Fluo3-AM and the Fluo3 fluorescence determined by flow cytometry. Prior to determination of Fluo3-fluorescence, erythrocytes were incubated in Ringer solution without or with

Fig. 4. Effect of gedunin 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 line) presence of 24 μM gedunin. B,C. Original dot plots of annexin V binding as a function of forward scatter following exposure for 48 h to Ringer solution without (B) and with (C) presence of 24 μM gedunin. D. Arithmetic means $\pm$ SEM of erythrocyte annexin-V-binding ($n = 8$) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of gedunin (2–24 μM). ** ($p < 0.01$), *** ($p < 0.001$) indicate significant difference from the absence of gedunin (ANOVA).

gedunin (2–24 μM). As shown in Fig. 3, a 48 h exposure of human erythrocytes to gedunin increased Fluo3 fluorescence, an effect reaching statistical significance at 12 μM gedunin concentration. Thus, gedunin increased cytosolic Ca^{2+} concentration.

An increase of $[\text{Ca}^{2+}]_i$ is known to stimulate cell membrane phospholipid scrambling leading to phosphatidylserine translocation to the erythrocyte surface. In order to identify phosphatidylserine exposing erythrocytes, annexin-V-binding was determined in flow cytometry. As shown in Fig. 4, a 48 h exposure to gedunin increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 12 μM gedunin. Thus, gedunin triggered erythrocyte cell membrane scrambling with phosphatidylserine translocation to the cell surface.

In order to quantify the effect of gedunin on hemolysis, the percentage of hemolysed erythrocytes was estimated from hemoglobin concentration in the supernatant. As a result, the percentage of hemolysed erythrocytes approached $0.21 \pm 0.07\%$, $0.86 \pm 0.50\%$, $1.2 \pm 0.85\%$, $3.4 \pm 1.6\%$ and $5.2 \pm 1.6\%$ following exposure of erythrocytes for 48 h to 0, 2, 6, 12 and 24 μM gedunin ($n = 4$).

A further series of experiments tested whether the gedunin induced cell membrane scrambling required entry of extracellular Ca^{2+} . To this end, erythrocytes were exposed to 24 μM gedunin for 48 h in the presence and in the nominal absence of extracellular Ca^{2+} . As shown in Fig. 5, the effect of gedunin on annexin-V-binding was virtually abrogated in the nominal absence of Ca^{2+} . Thus, the effect of gedunin was mainly if not exclusively due to Ca^{2+} entry.

Fig. 5. Effect of Ca^{2+} withdrawal on gedunin-induced annexin-V-binding. Arithmetic means $\pm$ SEM ($n = 4$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 24 μM gedunin in the presence (left bars) and absence (right bars) of calcium. *** ($p < 0.001$) indicates significant difference from the respective values in absence of gedunin, ### ($p < 0.001$) indicates significant difference from the respective value in the presence of Ca^{2+} (ANOVA).

Additional experiments were made on the effect of gedunin on ceramide formation. Following a 48 hour incubation with or without 24 μM gedunin, the ceramide abundance at the erythrocyte surface was 13.4 ± 3.1 ($n = 4$) in the absence and 13.8 ± 2.6 ($n = 4$) in the presence of gedunin, values not significantly different.

Discussion

The present study discloses a novel effect of gedunin, i.e. stimulation of erythrocyte cell membrane scrambling leading to phosphatidylserine translocation to the erythrocyte surface. Treatment of human erythrocytes with gedunin further decreased cell volume and increased cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). The concentrations required for those effects were similar to those encountered *in vivo* [14].

Erythrocytes express HSP 90 [67, 68] and the HSP 90 inhibitor geldanamycin has previously been shown to trigger eryptosis [60]. To the best of our knowledge this is the first demonstration that gedunin increases cytosolic $[\text{Ca}^{2+}]_i$. Notably, both, HSP90 and transient receptor potential channels, interact with XPORT (exit protein of rhodopsin and TRP) [71] and HSP90 may thus, at least in theory, modify the function of the erythrocyte cation channel, which involves TRPC6 [15]. The increase of $[\text{Ca}^{2+}]_i$ presumably accounts for the erythrocyte shrinkage. Increased $[\text{Ca}^{2+}]_i$ activates Ca^{2+} sensitive K^+ channels [15] followed by K^+ exit, cell membrane hyperpolarisation, Cl^- exit and thus cellular loss of KCl with osmotically obliged water [16]. The decrease of cell volume during eryptosis counteracts swelling of defective erythrocytes, which may otherwise lead to rupture of the erythrocyte membrane with subsequent cellular release, glomerular filtration and intratubular precipitation of hemoglobin [72].

Stimulation of Ca^{2+} entry may possibly contribute to the stimulation of apoptosis by gedunin. Increased $[\text{Ca}^{2+}]_i$ is a well known trigger of apoptosis in nucleated cells [73-75]. Further experiments in nucleated cells are warranted to explore whether gedunin increases $[\text{Ca}^{2+}]_i$ similarly in tumor cells and, if so, whether this effect participates in the triggering of apoptosis.

The stimulation of cell membrane scrambling with phosphatidylserine exposure at the cell surface may be favourable during infection with the malaria pathogen *Plasmodium falciparum*. In view of its effect on noninfected cells gedunin may accelerate suicidal death of infected erythrocytes [76]. The intraerythrocytic parasite activates several channels including Ca^{2+} -permeable erythrocyte cation channels [77, 78]. The channels provide the intraerythrocytic pathogen with nutrients, Na^+ and Ca^{2+} and allow the disposal of waste products [78]. Intraerythrocyte survival of the pathogen thus requires activation of those channels [77, 78]. The Ca^{2+} entry through the Ca^{2+} -permeable cation channels triggers, however, eryptosis [76], thus leading to rapid clearance of the infected erythrocytes from

circulating blood [15]. Accordingly, Ca^{2+} entry and subsequent eryptosis limits the life span of infected erythrocytes and thus counteracts parasitemia [76]. Along those lines several genetic disorders predisposing to accelerated eryptosis, such as sickle-cell trait, beta-thalassemia-trait, homozygous Hb-C and G6PD-deficiency [15] are known to confer partial resistance to malaria [79-81], even though the partial resistance may involve further mechanisms other than eryptosis [82-85].

In addition to genetic erythrocyte disorders, several clinical conditions and drugs stimulating eryptosis have been shown to favourably influence the clinical course of malaria, such as iron deficiency [86], lead [87], chlorpromazine [88] and inhibition of NO synthase [89].

On the other hand, stimulation of eryptosis may lead to anemia. Phosphatidylserine at the surface of eryptotic cells binds to the respective receptors of phagocytosing cells thus leading to rapid removal of the suicidal erythrocytes from circulating blood [15]. As soon as accelerated loss of erythrocytes during stimulated eryptosis cannot be matched by similarly enhanced formation of new erythrocytes, anemia develops [15]. Phosphatidylserine exposing erythrocytes may further impede microcirculation [90-95], as they adhere to endothelial CXCL16/SR-PSO [91]. Phosphatidylserine exposing erythrocytes may further stimulate blood clotting and thrombosis [90, 96, 97].

Whether or not gedunin and/or HSP90 inhibition leads to anemia and/or deranged microcirculation, remains to be established. In HSP90 knockout mice enhanced antibody production to a T-dependent antigen and enhanced MHC class II antigen presentation to T helper cells by dendritic cells has been observed [98]. To the best of our knowledge, nothing is hitherto known about anemia, microcirculation and thrombosis in those mice.

Conclusion

Gedunin stimulates Ca^{2+} entry leading to cell shrinkage and cell membrane scrambling of erythrocytes, hallmarks of suicidal erythrocyte death or eryptosis.

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