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Original Paper

Stimulation of Eryptosis by Cryptotanshinone

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Key Words
Phosphatidylserine • Cryptotanshinone • Calcium • Cell volume • Eryptosis

Abstract
Background/Aims: Cryptotanshinone, a component of *Salvia miltiorrhiza Bunge* roots, may trigger suicidal death or apoptosis of tumor cells and has thus been recommended for the prevention and treatment of malignancy. On the other hand, Cryptotanshinone has been shown to counteract apoptosis of neurons and hepatocytes. Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis, a suicidal death characterized by cell shrinkage and phosphatidylserine translocation to the erythrocyte surface. Eryptosis may be triggered by increase of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$). The present study explored whether Cryptotanshinone stimulates eryptosis. ***Methods:*** Forward scatter was taken as measure of cell volume, annexin V binding for identification of phosphatidylserine-exposing erythrocytes and Fluo3-fluorescence for determination of $[Ca^{2+}]_i$. ***Results:*** A 48 h exposure of human erythrocytes to Cryptotanshinone (10 μM) was followed by significant decrease of forward scatter, significant increase of the percentage annexin-V-binding cells and significant increase of $[Ca^{2+}]_i$. The effect of Cryptotanshinone (1 μM) on annexin-V-binding was virtually abrogated by removal of extracellular Ca^{2+}. ***Conclusion:*** Cryptotanshinone is a powerful stimulator of suicidal erythrocyte death or eryptosis, which is effective mainly, if not exclusively, by stimulation of Ca^{2+} entry.

Introduction

Cryptotanshinone is a terpenoid isolated from the roots of *Salvia miltiorrhiza Bunge*, which have been used in traditional Chinese medicine for treatment of a wide variety of clinical conditions [1-4]. Cryptotanshinone has been shown to be particularly effective in the

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prevention and treatment of malignancy [1]. The efficacy of Cryptotanshinone in cancer has been attributed to stimulation of suicidal cell death or apoptosis [5-16]. The proapoptotic effect of Cryptotanshinone has partially been explained by sensitisation to TRAIL [15, 17]. On the other hand, Cryptotanshinone has been shown to counteract apoptosis in some cell types [18-23].

In analogy to apoptosis of nucleated cells, erythrocytes may enter suicidal death or eryptosis, which is characterized by cell shrinkage and translocation of phosphatidylserine from the cell interior to the cell surface [24]. Stimulators of eryptosis include increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which activates Ca^{2+}-sensitive K^+ channels with subsequent K^+ exit, hyperpolarization, Cl^- exit and thus cellular loss of KCl and water leading to cell shrinkage [25]. Increased $[Ca^{2+}]_i$ triggers in addition phospholipid scrambling of the cell membrane with phosphatidylserine exposure at the erythrocyte surface [24]. Further triggers of eryptosis include ceramide formation [26], caspase activation [27-31] and deranged activities of AMP activated kinase AMPK [32], casein kinase 1α [33, 34], cGMP-dependent protein kinase [35], Janus-activated kinase JAK3 [36], protein kinase C [37], p38 kinase [38], PAK2 kinase [39] sorafenib sensitive kinases [40] as well as sunitinib sensitive kinases [41].

Eryptosis is triggered by a myriad of xenobiotics [26, 40-74] and excessive eryptosis is observed in a wide variety of clinical conditions, such as sepsis, malaria, sickle cell disease, Wilson's disease, iron deficiency, malignancy, metabolic syndrome, diabetes, renal insufficiency, hemolytic uremic syndrome, hyperphosphatemia and phosphate depletion [24, 75].

The present study explored, whether Cryptotanshinone stimulates or interferes with eryptosis. To this end, the effect of Cryptotanshinone on $[Ca^{2+}]_i$, cell volume and phosphatidylserine abundance at the surface of human erythrocytes have been determined utilizing flow cytometry.

Materials and Methods

Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes 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 (184/2003V). 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 Cryptotanshinone (Enzo, Lörrach, Germany) 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).

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).

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.

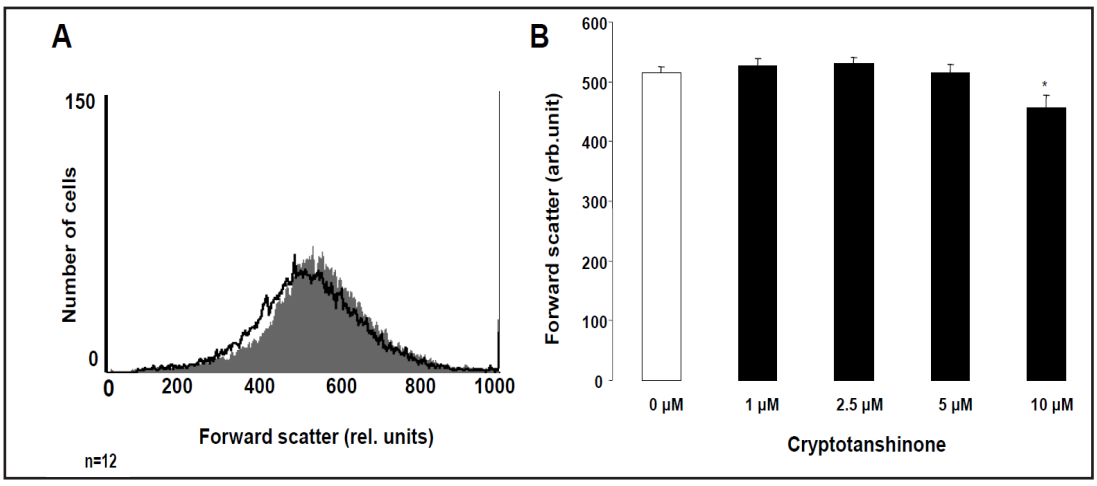

Fig. 1. Effect of Cryptotanshinone on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 10 µM Cryptotanshinone. B. Arithmetic means ± SEM (n = 8) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) Cryptotanshinone (1 - 10 µM). *($p<0.05$) indicates significant difference from the absence of Cryptotanshinone (ANOVA).

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 the influence of the terpenoid Cryptotanshinone on eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and phosphatidylserine translocation to the cell surface.

In order to estimate alterations of cell volume, forward scatter was determined in flow cytometry following incubation of the erythrocytes for 48 h in Ringer solution without or with Cryptotanshinone (1-10 µM). As illustrated in Fig. 1, a 48 h exposure to Cryptotanshinone was followed by a decrease of forward scatter, an effect reaching statistical significance at 10 µM Cryptotanshinone concentration. Accordingly, Cryptotanshinone treatment was followed by erythrocyte shrinkage.

Cell membrane phospholipid scrambling with phosphatidylserine translocation to the erythrocyte surface was quantified utilizing annexin-V-binding in flow cytometry. The annexin V binding was determined following incubation of the erythrocytes for 48 h in Ringer solution without or with Cryptotanshinone (1-10 µM). As illustrated in Fig. 2, a 48 h exposure to Cryptotanshinone was followed by an increase of the percentage annexin-V-binding erythrocytes, an effect reaching statistical significance at 5 µM Cryptotanshinone concentration. Thus, Cryptotanshinone triggered erythrocyte cell membrane scrambling with subsequent translocation of phosphatidylserine to the cell surface.

Since both, cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface are stimulated by increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), further experiments estimated the effect of Cryptotanshinone on $[Ca^{2+}]_i$. To this end,

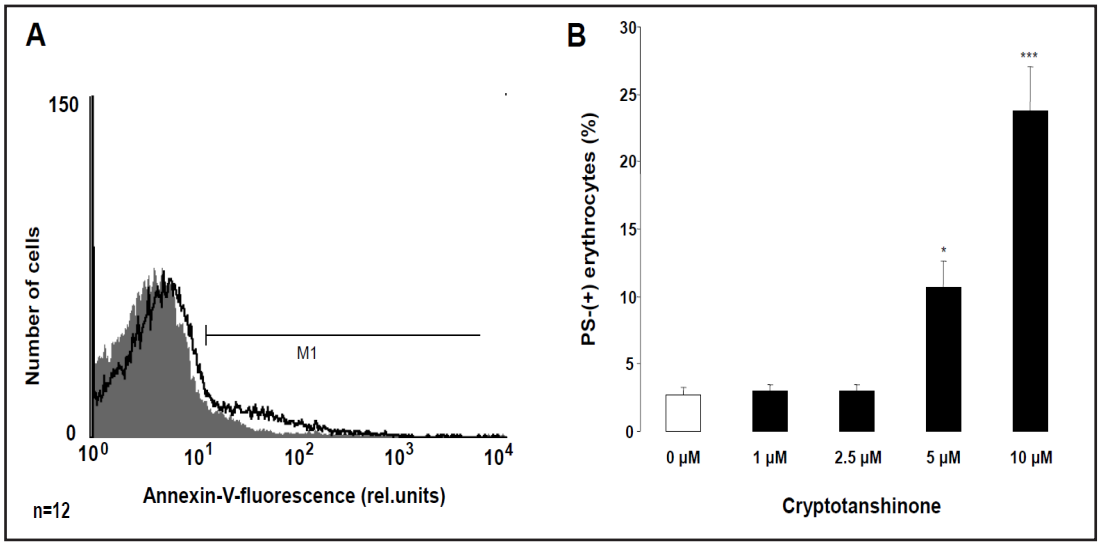

Fig. 2. Effect of Cryptotanshinone on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 10 µM Cryptotanshinone. B. Arithmetic means ± SEM (n = 8) of erythrocyte annexin-V-binding following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of Cryptotanshinone (1 - 10 µM). *(p<0.05), ***(p<0.001) indicate significant difference from the absence of Cryptotanshinone (ANOVA).

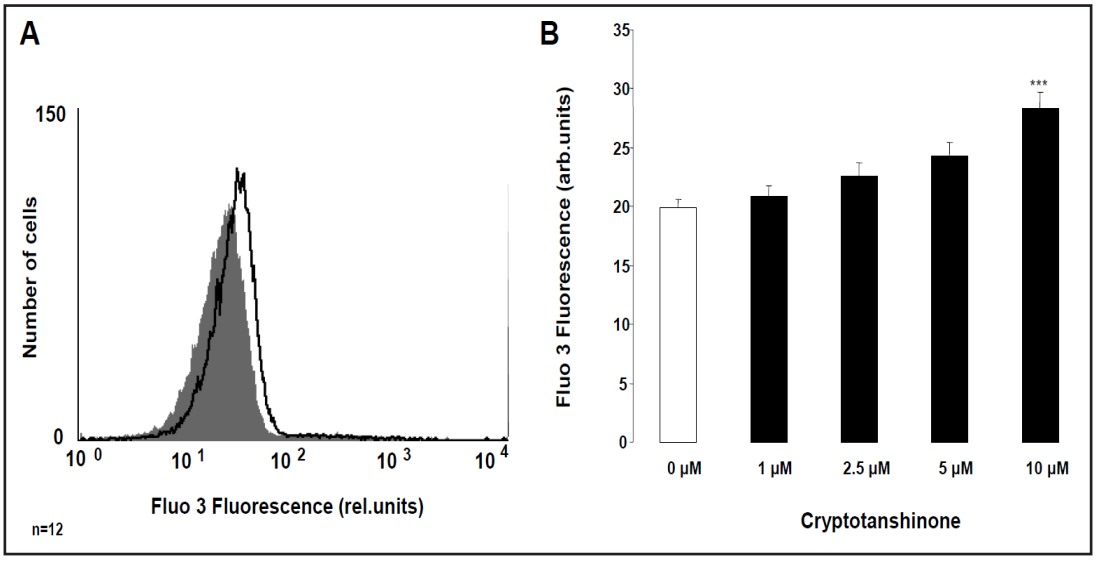

Fig. 3. Effect of Cryptotanshinone on erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 10 µM Cryptotanshinone. B. Arithmetic means ± SEM (n = 8) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) Cryptotanshinone (1 - 10 µM). ***(p<0.001) indicates significant difference from the absence of Cryptotanshinone (ANOVA).

erythrocytes were loaded with Fluo3-AM and the Fluo3 fluorescence determined by flow cytometry. Prior to determination of Fluo3-fluorescence, erythrocytes were incubated for 48 h in Ringer solution without or with Cryptotanshinone (1-10 µM). As shown in Fig. 3, exposure of the erythrocytes to Cryptotanshinone was followed by an increase of Fluo3 fluorescence, an effect reaching statistical significance at 10 µM Cryptotanshinone concentration. Thus, Cryptotanshinone increased cytosolic Ca^{2+} concentration.

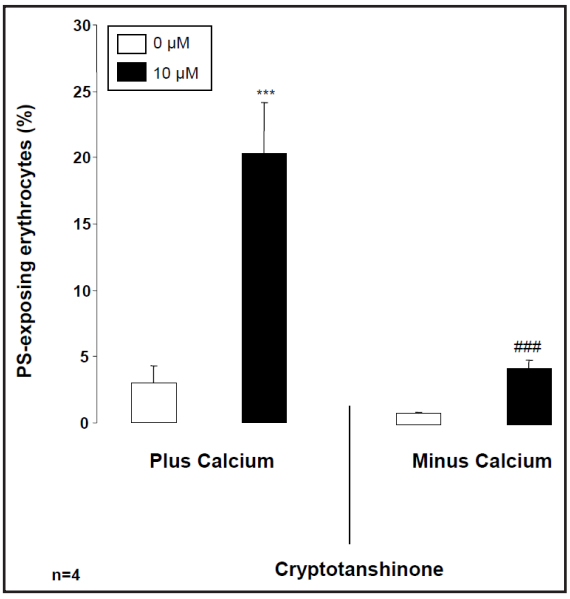

Fig. 4. Effect of Ca^{2+} withdrawal on Cryptotanshinone- induced annexin-V-binding. Arithmetic means ± SEM (n = 4) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 10 µM Cryptotanshinone in the presence (left bars, Plus Calcium) and absence (right bars, Minus Calcium) of calcium. ***($p<0.001$) indicates significant difference from the respective values in absence of Cryptotanshinone, ### ($p<0.001$) indicates significant difference from the respective value in the presence of Ca^{2+} (ANOVA).

A further series of experiments explored, whether the Cryptotanshinone induced cell membrane scrambling required entry of extracellular Ca^{2+}. To this end, erythrocytes were exposed for 48 h to 10 µM Cryptotanshinone in the presence or nominal absence of extracellular Ca^{2+}. As shown in Fig. 4, the effect of Cryptotanshinone on annexin-V-binding was significantly blunted and virtually abrogated in the nominal absence of Ca^{2+}. In the nominal absence of Cryptotanshinone the percentage of annexin V binding erythrocytes was not significantly different between presence and absence of Cryptotanshinone. Thus, Cryptotanshinone was effective mainly, if not exclusively, through stimulation of Ca^{2+} entry.

Discussion

The present study reveals that Cryptotanshinone stimulates eryptosis, the suicidal death of erythrocytes. Specifically, Cryptotanshinone triggered erythrocyte shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The Cryptotanshinone concentrations (10 µM) required for those effects were similar to those determined in Cryptotanshinone treated animals [76].

Cryptotanshinone triggered cell shrinkage presumably by increasing entry of extracellular Ca^{2+} with subsequent increase of $[Ca^{2+}]_i$, activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarisation, Cl^- exit and thus cellular loss of KCl with osmotically obliged water [25]. Exit of cellular KCl with water counteracts erythrocyte swelling, which may lead to hemolysis with subsequent release of hemoglobin. The hemoglobin released during hemolysis may be filtered in renal glomeruli and subsequently precipitate in the acidic lumen of renal tubules [77].

Increase of $[Ca^{2+}]_i$ further largely accounts for the stimulation of cell membrane scrambling by Cryptotanshinone. Apparently, Cryptotanshinone stimulates Ca^{2+} entry and its effect on cell membrane scrambling requires the presence of extracellular Ca^{2+}.

The ability of cryptotanshinone to trigger eryptosis is similar to that of tanshinone II [61]. Both, Cryptotanshinone [1] and Tanshinone [78] are utilized in the prevention and treatment of malignancy. Tanshinone [79-81] and Cryptotanshinone [2, 3] further exert several beneficial cardiovascular effects. Salvia plants have anti-oxidant, antimicrobial, antiplasmodial, analgesic, antipyretic, anticancer, anti-inflammatory and antinociceptive activities [82].

Stimulation of eryptosis fosters elimination of defective erythrocytes and may thus protect against untoward effects of hemolysis [24]. The removal of phosphatidylserine

exposing erythrocytes is particularly important during the course of malaria [83]. The intraerythrocytic parasite triggers eryptosis of infected erythrocytes [83] by activation of ion channels including the Ca^{2+}-permeable erythrocyte cation channels [84, 85]. The channel activity is required for the intraerythrocytic survival of the pathogen [84, 85], as the channels mediate entry of nutrients, Na^+ and Ca^{2+} as well as disposal of intracellular waste products [85]. Ca^{2+} entry through the Ca^{2+}-permeable cation channels triggers, however, eryptosis leading to rapid clearance of the infected erythrocytes from circulating blood [83]. Thus, eryptosis counteracts parasitemia by triggering elimination of the infected erythrocyte and thus of the pathogen [83]. Along those lines, genetic disorders sensitizing erythrocytes to eryptosis, such as sickle-cell trait, beta-thalassemia-trait, homozygous Hb-C and G6PD-deficiency [24, 86] are associated with a relatively mild clinical course of malaria [24, 87-89]. Moreover, clinical conditions with enhanced eryptosis, such as iron deficiency [90-95], and eryptosis stimulating drugs, such as lead [96], chlorpromazine [97] or NO synthase inhibitors [98] favourably influence the clinical course of malaria. Thus, at least in theory, Cryptotanshinone may similarly decrease parasitemia in malaria. Notably, *Salvia* is used for the treatment of malaria [82]. The efficacy may, however, at least in part be due to a more direct toxic effect on plasmodia [82].

Excessive stimulation of eryptosis may, however, result in anemia. Phagocytosis of phosphatidylserine exposing eryptotic erythrocytes leads to rapid removal of those cells and thus to a decrease of erythrocytes in circulating blood [24]. To the extent that the accelerated clearance of erythrocytes during stimulated eryptosis cannot be compensated by a similarly accelerated formation of new erythrocytes, anemia develops [24]. Phosphatidylserine exposing erythrocytes further adhere to endothelial CXCL16/SR-PSO [99] and stimulate blood clotting and thrombosis [100-102]. Thus, excessive eryptosis does interfere with microcirculation [99, 100, 103-106]. The effect of Cryptotanshinone on anemia and microcirculation may be augmented by other xenobiotics triggering eryptosis or in clinical disorders associated with enhanced eryptosis, such as renal insufficiency, diabetes or sepsis (see introduction).

Conclusion

The exposure of human erythrocytes to Cryptotanshinone stimulates Ca^{2+} entry, erythrocyte shrinkage and erythrocyte cell membrane scrambling. Thus, Cryptotanshinone triggers eryptosis, the suicidal erythrocyte death.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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