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Original Paper

Induction of Programmed Erythrocyte Death by Gambogic Acid

Adrian Lupescu* Kashif Jilani* Christine Zelenak Mohanad Zbidah Nazneen Shaik Florian Lang

Department of Physiology, University of Tuebingen, Tuebingen, *contributed equally and thus share the first authorship

Key Words
Phosphatidylserine • Gambogic acid • Calcium • Cell volume • Eryptosis

Abstract
Gambogic acid, a xanthone from *Garcinia hanburyi,* stimulates apoptosis and has thus anticancer potency. Similar to apoptosis of nucleated cells, erythrocytes may undergo apoptosis-like suicidal death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling leading to phosphatidylserine-exposure at the cell surface. Eryptosis could be triggered by increase of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$), ceramide formation, ATP-depletion and caspase activation. The present study explored, whether gambogic acid triggers eryptosis of human erythrocytes. $[Ca^{2+}]_i$ was estimated utilizing Fluo-3 fluorescence, cell volume from forward scatter, phosphatidylserine-exposure from annexin-V-binding, hemolysis from hemoglobin release, ceramide abundance utilizing antibodies, and cytosolic ATP with luciferin–luciferase. A 48 h exposure to gambogic acid (500 nM) significantly increased $[Ca^{2+}]_i$, stimulated ceramide formation, decreased forward scatter and increased annexin-V-binding. Gambogic acid exposure was followed by a slight but significant increase of hemolysis. Gambogic acid did not significantly modify cytosolic ATP-concentration. Removal of extracellular Ca^{2+} slightly, but significantly blunted the effect of gambogic acid (500 nM) on annexin-V-binding. The present observations disclose a novel effect of gambogic acid, i.e. stimulation of suicidal death of human erythrocytes or eryptosis, paralleled by Ca^{2+}-entry, ceramide formation, cell shrinkage and phosphatidylserine-exposure.

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Introduction

Gambogic acid, a xanthone from *Garcinia hanburyi*, is a nutraceutical with anticancer, anti-HIV-1, antibacterial, anti-inflammatory and neurotrophic activities [1-4]. Its anticancer activity presumably results from its ability to induce apoptosis [4-7]. Gambogic acid induces

Prof. Dr. Florian Lang
Physiologisches Institut, der Universität Tübingen
Gmelinstr. 5, D-72076 Tübingen (Germany)
Tel. +49 7071 29 72194, Fax +49 7071 29 5618
E-Mail florian.lang@uni-tuebingen.de

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apoptosis of a wide variety of cells including oral squamous cell carcinoma [8], gastric cancer cells [9-12], cholangiocarcinoma cells [2], hepatoma cells [13, 14], pancreatic cancer cells [15], lung cancer cells [16], breast cancer cells [17], prostate cancer cells [18], glioblastoma cells [19], osteosarcoma cells [20], Jurkat cells [21, 22], K562 cells [23] and A549 cells [24]. Gambogic acid is effective by interfering with the expression of Bax and Bcl-2 proteins [12, 17, 25, 26], inhibition of SRC-3 [23], inactivation of the Akt pathway [23] stimulation of the transferrin receptor [16, 27, 28], interference with nucleophosmin and nucleoporins [21], interaction with Hsp90 [29], deregulation of NF-κB [29], induction of p21(Waf1/CIP1) expression [30], inhibition of STAT3 phosphorylation [31], inhibition of survivin [11], proteasome inhibition [32], down-regulation of mdm2 expression [30, 33], reactive oxygen species accumulation [13], interaction with K^+ channel activity [34], suppression of vascular endothelial growth factor signaling [18], inhibition of angiogenesis [18], and activation of T lymphocytes [35].

Similar to apoptosis of nucleated cells, suicidal erythrocyte death or eryptosis is paralleled by cell membrane scrambling and cell shrinkage [36]. Eryptosis is elicited by Ca^{2+} entry through Ca^{2+}-permeable cation channels [37, 38]. The subsequent increase of cytosolic Ca^{2+} concentration results in activation of Ca^{2+}-sensitive K^+ channels [39] with K^+ exit, hyperpolarization, Cl^- exit and thus cellular KCl loss together with osmotically obliged water, events leading to cell shrinkage [40]. The increased cytosolic Ca^{2+} concentration is further followed by cell membrane scrambling with phosphatidylserine exposure at the cell surface [41]. The Ca^{2+} sensitivity of erythrocyte cell membrane scrambling is increased by ceramide, which similarly stimulates eryptosis [42]. Further triggers of eryptosis include energy depletion [43] and activation of caspases [44-48]. Eryptosis is further influenced by AMP activated kinase AMPK [38], cGMP-dependent protein kinase [49] and Janus-activated kinase JAK3 [50].

The present study explored, whether gambogic acid triggers eryptosis and, if so, to elucidate the underlying mechanisms.

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 gambogic acid (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).

FACS analysis of annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 50 µl cell suspension were 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 in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

Confocal microscopy and immunofluorescence

For the visualization of eryptotic erythrocytes, 4 µl of erythrocytes, incubated in the respective experimental solutions, were stained with FITC-conjugated Annexin-V-Fluos (1:100 dilution; ImmunoTools, Friesoythe, Germany) in 200 µl Ringer solution containing 5 mM $CaCl_2$. The erythrocytes were washed twice and finally resuspended in 50 µl of Ringer solution containing 5 mM $CaCl_2$. The cell suspension (20 µl) was mounted with Prolong Gold antifade reagent (Invitrogen, Darmstadt, Germany) onto a glass slide, covered

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with a coverslip and images were subsequently taken on a Zeiss LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss MicroImaging, Oberkochen, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

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 2 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 in fluorescence channel FL-1 in FACS analysis.

Determination of intracellular ATP concentration

For determination of intracellular erythrocyte ATP, 90 µl of erythrocyte pellets were incubated for 48 h at 37°C in Ringer solution with or without gambogic acid and in Ringer solution with or without extracellular calcium (final hematocrit 5%). Additionally, erythrocytes were also incubated in glucose depleted Ringer solution as a positive control. All subsequent manipulations were performed at 4°C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of $HClO_4$ (5%). After centrifugation, an aliquot of the supernatant (400 µl) was adjusted to pH 7.7 by addition of saturated $KHCO_3$ solution. After dilution of the supernatant, the ATP concentrations of the aliquots were determined utilizing the luciferin–luciferase assay kit (Roche Diagnostics) on a luminometer (Berthold Biolumat LB9500, Bad Wildbad, Germany) according to the manufacturer's protocol. ATP concentrations are expressed in mmol/l cytosol of erythrocytes.

Determination of ceramide formation

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation with and without gambogic acid, cells were stained for 1 h 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 1:5. 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 in FL-1.

Measurement of hemolysis

For the determination of hemolysis the samples were centrifuged (3 min at 400 g, room temperature) after incubation, and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of 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

In order to estimate cytosolic Ca^{2+} activity in erythrocytes, the erythrocytes were loaded with Fluo-3 and the Fluo-3 fluorescence determined in FACS analysis. As illustrated in Fig. 1, treatment of human erythrocytes with gambogic acid resulted in an increase of Fluo-3 fluorescence. The effect reached statistical significance at 500 nM gambogic acid concentrations. Thus, gambogic acid increased cytosolic Ca^{2+} concentration.

An increase of cytosolic Ca^{2+} concentration is known to trigger erythrocyte shrinkage by activation of Ca^{2+}-sensitive K^+ channels with subsequent exit of KCl paralleled by osmotically

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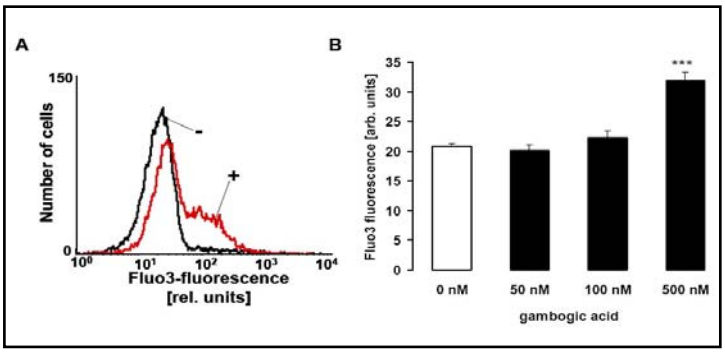

Fig. 1. Effect of gambogic acid on erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo-3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) presence of 500 nM gambogic acid. B. Arithmetic means ± SEM (n = 10) of the normalized geo means (geometric mean of the histogram in arbitrary units) of Fluo-3 fluorescence in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) gambogic acid (50-500 nM). *** (p<0.001) indicates significant difference from the absence of gambogic acid (ANOVA).

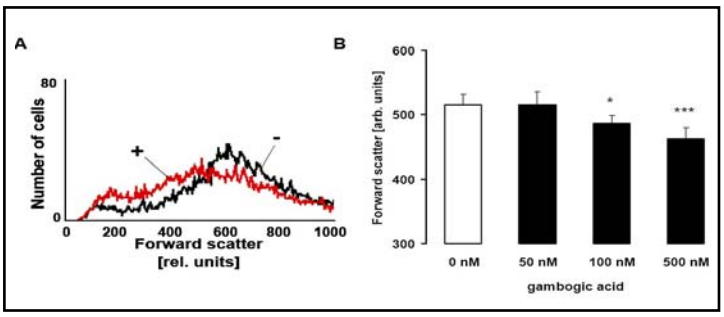

Fig. 2. Effect of gambogic acid on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) presence of 500 nM gambogic acid. B. Arithmetic means ± SEM (n = 10) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) gambogic acid (50-500 nM). *, *** (p<0.05, p<0.001) indicates significant difference from the absence of gambogic acid (ANOVA).

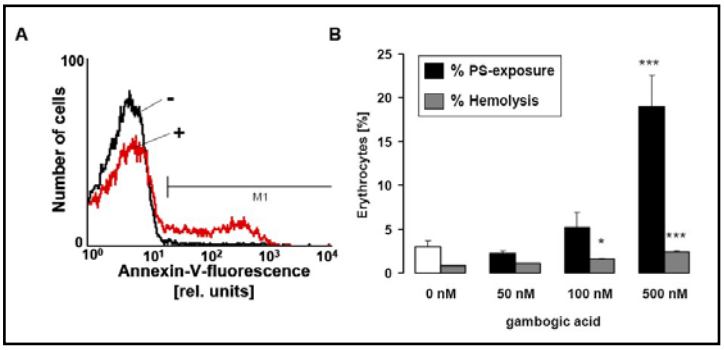

Fig. 3. Effect of gambogic acid on phosphatidylserine exposure and hemolysis. A. Original histogram of annexin-V binding of erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) presence of 500 nM gambogic acid. B. Arithmetic means ± SEM of erythrocyte annexin-V-binding (n = 10) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of gambogic acid (50-500 nM). For comparison, arithmetic means ± SEM (n = 4) of the percentage of hemolysis is shown as grey bars. * (p<0.05), *** (p<0.001) indicates significant difference from the absence of gambogic acid (ANOVA).

obliged water. To test, whether gambogic acid exposure indeed leads to cell shrinkage, cell volume was estimated utilizing forward scatter in FACS analysis. As shown in Fig. 2, gambogic acid treatment decreased forward scatter, an effect statistically significant at 100 nM gambogic acid.

An increase of cytosolic Ca^{2+} activity is further known to trigger cell membrane scrambling with phosphatidylserine exposure at the cell surface. In order to test whether gambogic acid treatment is indeed followed by cell membrane scrambling, phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by FACS analysis. As illustrated in Fig. 3, a 48 h exposure to gambogic acid increased the percentage of annexin-

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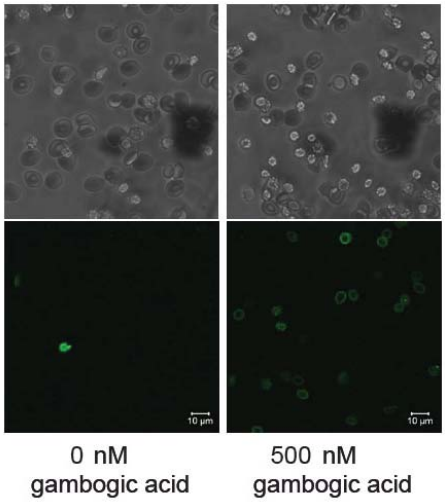


Fig. 4. Phosphatidylserine exposing erythrocytes following gambogic acid exposure. Confocal microscopy of light microscopy (upper panels) and FITC-dependent fluorescence (lower panels) of human erythrocytes stained with FITC-conjugated Annexin-V-Fluos following 48 hours incubation in Ringer solution without (left panels) and with (right panels) 500 nM gambogic acid.

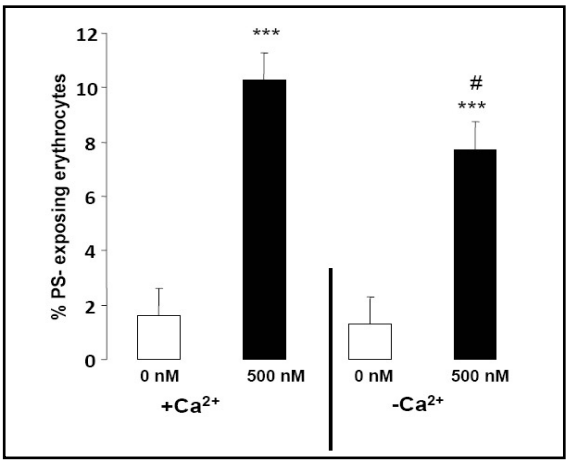
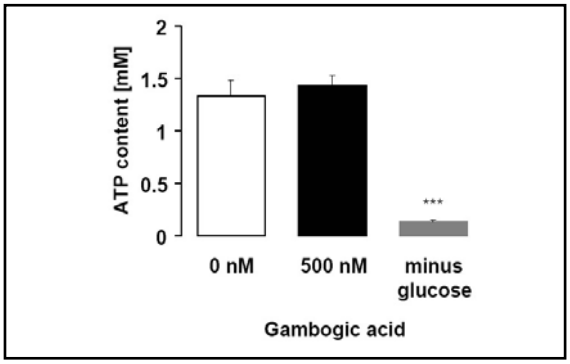


Fig. 5. Effect of Ca^{2+} withdrawal on gambogic acid-induced annexin-V-binding. Arithmetic means ± SEM (n = 4) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 500 nM gambogic acid in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of calcium. *** ($p<0.001$) indicates significant difference from the absence of gambogic acid (ANOVA) # ($p<0.05$) indicates significant difference from the respective values in the presence of Ca^{2+}.

Fig. 6. Effect of gambogic acid on erythrocyte cytosolic ATP content. Arithmetic means ± SEM (n = 4) of the ATP concentration after a 48 h incubation in Ringer solution without (white bar) or with (black bar) 500 nM gambogic acid, or in glucose-depleted Ringer solution (grey bar, minus glucose). *** ($p<0.001$) indicates significant difference from control (absence of gambogic acid and presence of glucose) (ANOVA).



V-binding erythrocytes, an effect reaching statistical significance at 500 nM gambogic acid. The effect of gambogic acid on annexin-V-binding was confirmed by confocal microscopy (Fig. 4).

Further experiments explored whether gambogic acid triggered hemolysis, which was estimated from hemoglobin release into the supernatant. As shown in Fig. 3, exposure of erythrocytes for 48 h to gambogic acid indeed increased the hemoglobin concentration in the supernatant, an effect reaching statistical significance at 100 nM (Fig. 3 B). The percentage of hemolysed erythrocytes was, however, one order of magnitude smaller than the percentage of phosphatidylserine exposing cells.

Another series of experiments explored whether the gambogic acid induced cell membrane scrambling was dependent on Ca^{2+} entry. To this end, erythrocytes were exposed to 500 nM gambogic acid in the presence or in the nominal absence of extracellular Ca^{2+}. As shown in Fig. 5, the effect of gambogic acid on annexin-V-binding was significantly blunted in the nominal absence of Ca^{2+}. However, even in the nominal absence of Ca^{2+}, gambogic acid still significantly stimulated annexin-V-binding. Accordingly, the stimulation of erythrocyte

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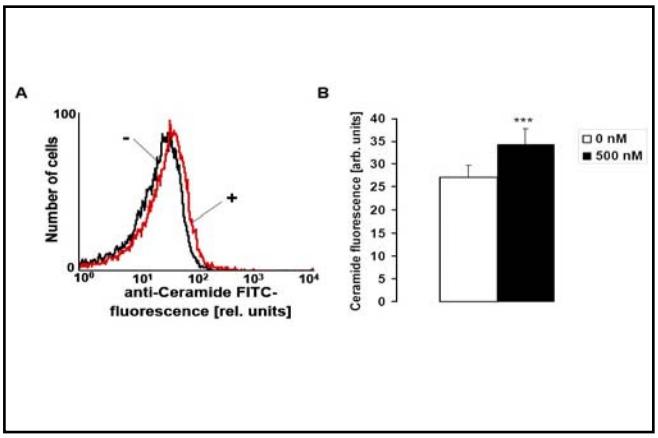

Fig. 7. Effect of gambogic acid on ceramide formation. A. Original histogram of anti-ceramide FITC-fluorescence in erythro-cytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) presence of 500 nM gambogic acid. B. Arithmetic means ± SEM (n = 10) of ceramide abundance after a 48 h incubation in Ringer solution without (white bar) or with (black bars) gambogic acid (500 nM). *** (p <0.001) indicates significant difference from control (absence of gambogic acid) (t test).

membrane scrambling by gambogic acid must have involved mechanisms in addition to Ca^{2+} entry.

In order to test whether gambogic acid leads to cellular energy depletion, cytosolic ATP concentration was determined utilizing a luciferin–luciferase assay kit. As shown in Fig. 6, the cytosolic ATP concentration in erythrocytes was not significantly modified by a 48 h exposure to 500 nM gambogic acid. As a positive control, erythrocytes were incubated in glucose-free Ringer, which significantly decreased cytosolic ATP concentration (Fig. 6). Thus, in contrast to glucose depletion gambogic acid exposure was not followed by a decline of intracellular ATP.

An additional series of experiments explored the effect of gambogic acid treatment on the formation of ceramide. Ceramide abundance was determined utilizing FITC-labeled anti-ceramide antibodies. As illustrated in Fig. 7, gambogic acid significantly increased ceramide-dependent fluorescence.

Discussion

The present observations reveal that gambogic acid triggers eryptosis, the suicidal death of erythrocytes. Specifically, gambogic acid exposure elicits erythrocyte membrane scrambling and leads to erythrocyte shrinkage. The concentration required for the effect on cell membrane scrambling is in the range of the concentrations effective against tumor growth *in vivo*, which has been observed at *in vivo* dosages of 4 mg/kg (6 µmol/kg) and 8 mg/kg (13 µmol/kg) [51].

Gambogic acid increases cytosolic Ca^{2+} activity, an effect presumably due to activation of non-selective cation channels. The molecular identity of the Ca^{2+} permeable erythrocyte cation channels is still incompletely understood but involves TRPC6 [37]. The cation channels have previously been shown to be activated by oxidative stress [52].

The increase of cytosolic Ca^{2+} activity is expected to activate Ca^{2+} - sensitive K^+ channels [39, 53] with subsequent exit of K^+ following its chemical gradient, cell membrane hyperpolarisation, Cl^- exit and thus cellular loss of KCl with osmotically obliged water [40]. Accordingly, the increase of cytosolic Ca^{2+} activity following gambogic acid treatment is expected to trigger cell shrinkage, which, according to forward scatter, was indeed a concequence of gambogic acid treatment.

As shown previously [41, 54, 55], an increase of cytosolic Ca^{2+} activity further stimulates cell membrane scrambling leading to phosphatidylserine exposure at the erythrocyte surface. The nominal absence of extracellular Ca^{2+} significantly blunted, but did not fully abrogate the scrambling effect of gambogic acid, indicating that gambogic acid must induce cell membrane scrambling by some additional mechanism(s). Gambogic acid obviously did not induce energy depletion, a known trigger of eryptosis [43].

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Gambogic acid did, however, increase the formation of ceramide, which has previously been shown to stimulate cell membrane scrambling in erythrocytes [42, 56]. Moreover, ceramide may trigger apoptosis of nucleated cells [57]. Excessive ceramide formation contributes to the pathophysiology of several clinical disorders including lung inflammation, fibrosis and infection [58], cystic fibrosis [59], cardiovascular disease [60, 61], Wilson's disease [62], multiple sclerosis [63], major depression [57], Parkinson's disease [64], Alzheimer's disease [57, 65, 66] and diabetes [67-69].

Eryptosis is triggered by a wide variety of xenobiotics [44, 50, 70-72, 72-79] and participates in the pathophysiology of diverse clinical disorders [36], such as diabetes [48, 80, 81], renal insufficiency [82], hemolytic uremic syndrome [83], sepsis [84], sickle cell disease [85]. malaria [62, 86-89], Wilson's disease [62], iron deficiency [90], phosphate depletion [91] and presumably metabolic syndrome [92].

Phosphatidylserine exposing erythrocytes adhere to endothelial CXCL16/SR-PSO [93]. The adhesion of phosphatidylserine exposing erythrocytes to the vascular wall may impair microcirculation [93-98]. Phosphatidylserine exposing erythrocytes further stimulate blood clotting [94, 99, 100]. Thus, excessive eryptosis may foster thrombosis. Moreover, the clearance of phosphatidylserine exposing erythrocytes from circulating blood may lead to anemia [36].

In conclusion, gambogic acid fosters Ca^{2+} entry and ceramide formation, effects triggering cell membrane scrambling and cell shrinkage, hallmarks of eryptosis. Thus, similar to its effect on nucleated cells, gambogic acid stimulates the suicidal death of erythrocytes.

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