Original Paper

Licochalcone A Induced Suicidal Death of Human Erythrocytes

Jasmin Egler Florian Lang

Department of Physiology, University of Tübingen, Tübingen, Germany

Key Words

Phosphatidylserine • Cell volume • Eryptosis • Oxidative stress • Calcium

Abstract

Background: The anti-inflammatory, immunomodulatory, and antimicrobial *Glycyrrhiza inflata* extract component licochalcone A triggers apoptosis of tumor cells and is thus considered for the treatment of malignancy. Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Signaling involved in the triggering of eryptosis includes Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), and ceramide. The present study explored, whether and how licochalcone A induces eryptosis. **Methods:** Human erythrocytes drawn from healthy individuals were exposed for 24 hours to 1–10 $\mu\text{g/ml}$ licochalcone A. Flow cytometry was subsequently employed to estimate phosphatidylserine exposure at the cell surface from annexin V binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, and ceramide utilizing specific antibodies. In addition, hemolysis was quantified from hemoglobin release. **Results:** Licochalcone A significantly increased the percentage of annexin-V-binding cells ($\geq 5 \mu\text{g/ml}$), significantly decreased forward scatter (2.5 – 5 $\mu\text{g/ml}$), significantly increased Fluo3-fluorescence ($\geq 7.5 \mu\text{g/ml}$), and significantly increased ceramide abundance (10 $\mu\text{g/ml}$). The effect of licochalcone on annexin-V-binding was not significantly modified, but hemolysis significantly enhanced by removal of extracellular Ca^{2+} . **Conclusions:** Licochalcone triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect independent from Ca^{2+} entry and presumably in part due to ceramide.

© 2015 The Author(s)
Published by S. Karger AG, Basel

Introduction

Licochalcone A, an anti-inflammatory [1–4], immunomodulatory [5, 6], and antimicrobial [3, 5, 7–9] polyphenol extracted from *Glycyrrhiza inflata* [1–3, 10], triggers apoptosis or inhibits proliferation and migration of tumor cells [11], including those of cervical cancer [12], oral squamous carcinoma [7, 9, 13–15], esophageal carcinoma [16], gastric cancer [4, 17, 18], hepatocellular carcinoma [2, 19], malignant pleural mesothelioma [20], bladder

cancer [21, 22], prostate tumor [23-25], ovarian carcinoma [26, 27], breast cancer [24], lung cancer [28] and leukemia [29]. Licochalcone A is thus considered for the treatment of malignancy [12, 30]. Licochalcone A is partially effective by inducing oxidative stress [17, 19, 21, 26], mitochondrial depolarization [20, 22], caspase activation [13, 14, 25, 26], inhibition of phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of mTOR signaling [12, 25, 28, 31], suppression of nuclear factor-kappa B (NF- κ B) [2, 9], interaction with the Jak/Stat pathway [29], suppression of cyclooxygenase (COX)-2 expression [4, 31], and inhibition of cytochrome P450 (CYP) enzymes [32].

On the other hand, licochalcone A has been shown exert cytoprotective effects by decreasing oxidative stress [33]. Licochalcone B and licochalcone D have been shown to counteract apoptosis of cardiomyocytes [34-36], an effect involving inhibition of inflammation and oxidative stress, suppression of NF- κ B/p65 and p38 MAPK signaling and activation of the AKT pathway [35-37]. Licochalcone A is further effective against atopic dermatitis [38], asthma [39, 40], and osteoporosis [10, 41]. Licochalcone A further inhibits the liver X receptor α and thus interferes with lipogenesis [37, 42-44].

Similar to licochalcone A, licochalcone F counteracts inflammation [45]. The proapoptotic and antitumor effect of licochalcone A is shared by licochalcone B [46, 47] and E [48, 49], and the antimicrobial effect by licochalcone E [50].

Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal death of erythrocytes which is characterized by cell shrinkage and by phospholipid scrambling of the cell membrane leading to phosphatidylserine translocation to the cell surface [51]. Stimulators of eryptosis include energy depletion, increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), ceramide, caspases, casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and p38 kinase [51]. Inhibitors of eryptosis include AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase and sorafenib/sunitinib sensitive kinases [51].

The present study explored whether licochalcone triggers eryptosis. To this end, human erythrocytes from healthy volunteers were treated with licochalcone and phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$ 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 24 h. Where indicated, erythrocytes were exposed to licochalcone A (Sigma Aldrich, Hamburg, Germany) at the indicated concentrations.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 100 μ l 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). 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".

Hemolysis

For the determination of hemolysis, the samples were centrifuged (10 min at 2000 rpm, room temperature) after incubation under the respective experimental conditions 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.

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 once 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.

Ceramide abundance

To determine the ceramide abundance at the erythrocyte surface, a monoclonal antibody was used. After incubation, cells were stained for 1 h at 37°C with 1 $\mu\text{g}/\text{ml}$ anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in phosphate-buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA) at a dilution of 1:10. After two washing steps with PBS-BSA, cells were stained for 30 min with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (BD Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. Samples were then analyzed by flow cytometric analysis at 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 may be differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study addressed the putative effect of licochalcone on eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface. Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding which was quantified by flow cytometry. The erythrocytes were analysed following a 24 hours incubation in Ringer solution without or with licochalcone (1 - 10 $\mu\text{g}/\text{ml}$). As illustrated in Fig. 1, a 24 hours exposure to licochalcone increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 5 $\mu\text{g}/\text{ml}$ licochalcone.

In order to estimate erythrocyte volume, forward scatter was determined by flow cytometry. Again, the measurement was made after a 24 hours incubation in Ringer solution without or with licochalcone (1 - 10 $\mu\text{g}/\text{ml}$). As shown in Fig. 2, exposure of erythrocytes to 2.5 or 5 $\mu\text{g}/\text{ml}$ licochalcone decreased erythrocyte forward scatter. No significant alterations of cell volume were observed at lower (1 $\mu\text{g}/\text{ml}$) and higher concentrations (7.5 and 10 $\mu\text{g}/\text{ml}$ of licochalcone).

Fluo3-fluorescence was taken as measure of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). As illustrated in Fig. 3, a 24 hours exposure to licochalcone increased the Fluo3-fluorescence, an effect reaching statistical significance at 7.5 $\mu\text{g}/\text{ml}$ licochalcone.

In order to test whether licochalcone-induced translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular Ca^{2+} , erythrocytes were incubated for 24 hours in the absence or presence of 5 or 10 $\mu\text{g}/\text{ml}$ licochalcone in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 4, removal of extracellular Ca^{2+} did not significantly modify the effect of 5 $\mu\text{g}/\text{ml}$ and 10 $\mu\text{g}/\text{ml}$ licochalcone. Thus, licochalcone-induced cell membrane scrambling was not dependent on entry of extracellular Ca^{2+} .

As shown in Fig. 5, following removal of extracellular Ca^{2+} , 5 or 10 $\mu\text{g}/\text{ml}$ licochalcone still tended to decrease forward scatter, an effect, however not reaching statistical significance.

Fig. 1. Effect of licochalcone on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of 10 µg/ml licochalcone. B. Arithmetic means $\pm$ SEM (n = 8) of erythrocyte annexin-V-binding (black bars) following incubation for 24 hours to Ringer solution without or with presence of licochalcone (1 - 10 µg/ml). *(p<0.05), *** (p<0.001) indicates significant difference from the absence of licochalcone (ANOVA).

Fig. 2. Effect of licochalcone on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of 10 µg/ml licochalcone. B. Arithmetic means $\pm$ SEM (n = 8) of the erythrocyte forward scatter (FSC) following incubation for 24 hours to Ringer solution without (white bar) or with (black bars) licochalcone (1 - 10 µg/ml). ** (p<0.01) indicates significant difference from the absence of licochalcone (ANOVA).

Fig. 3. Effect of licochalcone on erythrocyte Ca^{2+} activity. A. Original histogram of Fluo3-fluorescence in erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of licochalcone (10 µg/ml). B. Arithmetic means $\pm$ SEM (n = 8) of the Fluo3-fluorescence (arbitrary units) in erythrocytes exposed for 24 hours to Ringer solution without (white bar) or with (black bars) licochalcone (1 - 10 µg/ml). *** (p<0.001) indicates significant difference from the absence of licochalcone (ANOVA).

In order to quantify hemolysis, the hemoglobin concentration in the supernatant was determined by photometry. As shown in Fig. 6, a 24 hours incubation with licochalcone increased the percentage of hemolysed erythrocytes. Removal of extracellular Ca^{2+} did not significantly modify the effect of 5 µg/ml licochalcone on hemolysis. However, removal of

Fig. 4. Ca²⁺ insensitivity of licochalcone-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of licochalcone (5 and 10 µg/ml) in the presence (A) and absence (B) of extracellular Ca²⁺. C. Arithmetic means ± SEM (n = 8) of annexin-V-binding of erythrocytes after a 24 hours treatment with Ringer solution without (white bars) or with (black bars) licochalcone (5 or 10 µg/ml) in the presence (left bars, +Ca²⁺) and absence (right bars, -Ca²⁺) of Ca²⁺. *(p < 0.05), *** (p < 0.001) indicates significant difference from the absence of licochalcone.

Fig. 5. Impact of Ca²⁺ removal on licochalcone-induced alterations of erythrocyte volume. A,B. Original histograms of erythrocyte forward scatter following exposure for 24 hours to Ringer solution without (grey areas) and with (black lines) presence of licochalcone (10 µg/ml) in the presence (A) and absence (B) of extracellular Ca²⁺. C. Arithmetic means ± SEM (n = 8) of erythrocyte forward scatter after a 24 hours treatment with Ringer solution without (white bars) or with (black bars) licochalcone (5 or 10 µg/ml) in the presence (left bars, +Ca²⁺) and absence (right bars, -Ca²⁺) of Ca²⁺. *(p < 0.05), *** (p < 0.001) indicates significant difference from the absence of licochalcone.

Fig. 6. Impact of Ca²⁺ removal of licochalcone-induced hemolysis. Arithmetic means ± SEM (n = 4) of annexin-V-binding of erythrocytes after a 24 hours treatment with Ringer solution without (white bars) or with (black bars) licochalcone (5 or 10 µg/ml) in the presence (left bars, +Ca²⁺) and absence (right bars, -Ca²⁺) of Ca²⁺. *** (p < 0.001) indicates significant difference from the absence of licochalcone, ### (p < 0.001) indicates significant difference from the presence of Ca²⁺ (ANOVA).

extracellular Ca²⁺ significantly augmented the stimulating effect of 10 µg/ml licochalcone on hemolysis.

Ca²⁺ independent triggers of eryptosis include ceramide. Thus, specific antibodies have been employed to quantify the ceramide abundance at the erythrocyte surface. As illustrated

Fig. 7. Effect of licochalcone on erythrocyte ceramide abundance. A. Original histogram of ceramide abundance at the erythrocyte surface following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of licochalcone (10 µg/ml). B. Arithmetic means $\pm$ SEM ($n = 8$) of the ceramide abundance (arbitrary units) at the erythrocyte surface following exposure for 24 hours to Ringer solution without (white bar) or with (black bars) licochalcone (5 and 10 µg/ml). ***($p < 0.001$) indicates significant difference from the absence of licochalcone (ANOVA).

in Fig. 7, a 24 hours exposure to licochalcone (10 µg/ml) significantly increased the ceramide abundance.

Discussion

The present study discloses a novel effect of licochalcone, i.e. the triggering of suicidal erythrocyte death or eryptosis. Exposure of human erythrocytes to licochalcone results in cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentrations required for the effect are in the range of those required to induce apoptosis of tumor cells [17].

The effect of licochalcone on cell membrane scrambling and cell shrinkage was paralleled by increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Ca^{2+} entry from the extracellular space appears not to be required, however, for the effect of licochalcone on cell membrane scrambling. Removal of extracellular Ca^{2+} disrupted, however, the licochalcone induced cell shrinkage. An increase of $[\text{Ca}^{2+}]_i$ activates Ca^{2+} sensitive K^+ channels which contributes to or even accounts for the cell shrinkage due to K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [52]. Following an increase of licochalcone concentration to 7.5 or 10 µg/ml the licochalcone induced cell shrinkage disappeared despite an increasing $[\text{Ca}^{2+}]_i$. Possibly, licochalcone has a dual effect on erythrocyte cell volume, a Ca^{2+} dependent cell shrinkage and a Ca^{2+} independent cell swelling. Possibly due to the latter effect, licochalcone triggers hemolysis, an effect augmented by removal of extracellular Ca^{2+} . It is actually the purpose of the Ca^{2+} sensitive K^+ channels to counteract swelling of defective erythrocytes [51]. The phosphatidylserine exposure is followed by rapid clearance of the defective erythrocytes from circulating blood [51]. The preceding eryptosis is thus supposed to prevent hemolysis. The hemolysis would otherwise lead to release of hemoglobin, which is filtered in renal glomerula, precipitates in the acidic lumen of renal tubules and thus occludes nephrons [53].

Excessive eryptosis may, on the other hand, lead to anemia, if the formation of new erythrocytes cannot match the loss of eryptotic erythrocytes [51]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [54], stimulate blood clotting and trigger thrombosis [55-57]. Thus, triggering of eryptosis may impair microcirculation [55, 58-62].

It is expected that the sensitivity to the eryptotic effect of licochalcone is increased by clinical conditions associated with enhanced eryptosis, such as dehydration [63],

hyperphosphatemia [64] chronic kidney disease (CKD) [65-68], hemolytic-uremic syndrome [69], diabetes [70], hepatic failure [71], malignancy [51], sepsis [72], sickle-cell disease, beta-thalassemia, Hb-C and G6PD-deficiency [51], as well as Wilsons disease [73]. In those disorders lower licochalcone concentrations may be sufficient to trigger eryptosis. Moreover, eryptosis is stimulated by a wide variety of xenobiotics [63, 64, 66, 74-96]. Combination of licochalcone with those xenobiotics may lead to additive effects.

In conclusion, licochalcone triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect not depending on Ca^{2+} activity but paralleled by and presumably in part due to increased ceramide abundance.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Tanja Loch. The study was supported by the Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Tuebingen University.

Disclosure Statement

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

References

- 1 Dunlap TL, Wang S, Simmler C, Chen SN, Pauli GF, Dietz BM, Bolton JL: Differential Effects of Glycyrrhiza Species on Genotoxic Estrogen Metabolism: Licochalcone A Downregulates P450 1B1, whereas Isoliquiritigenin Stimulates It. *Chem Res Toxicol* 2015;28:1584-1594.
- 2 Tsai JP, Hsiao PC, Yang SF, Hsieh SC, Bau DT, Ling CL, Pai CL, Hsieh YH: Licochalcone A suppresses migration and invasion of human hepatocellular carcinoma cells through downregulation of MKK4/JNK via NF-kappaB mediated urokinase plasminogen activator expression. *PLoS One* 2014;9:e86537.
- 3 Zhao H, Jiang JT, Zheng QS: [Advance in studies on pharmacological effects of licochalcone A]. *Zhongguo Zhong Yao Za Zhi* 2013;38:3814-3818.
- 4 Park JM, Park SH, Hong KS, Han YM, Jang SH, Kim EH, Hahm KB: Special licorice extracts containing lowered glycyrrhizin and enhanced licochalcone A prevented *Helicobacter pylori*-initiated, salt diet-promoted gastric tumorigenesis. *Helicobacter* 2014;19:221-236.
- 5 Feldman M, Grenier D: Cranberry proanthocyanidins act in synergy with licochalcone A to reduce *Porphyromonas gingivalis* growth and virulence properties, and to suppress cytokine secretion by macrophages. *J Appl Microbiol* 2012;113:438-447.
- 6 Fontes LB, Dos Santos Dias D, de Carvalho LS, Mesquita HL, da Silva Reis L, Dias AT, Da Silva Filho AA, do Amaral Correa JO: Immunomodulatory effects of licochalcone A on experimental autoimmune encephalomyelitis. *J Pharm Pharmacol* 2014;66:886-894.
- 7 Kim JS, Park MR, Lee SY, Kim do K, Moon SM, Kim CS, Cho SS, Yoon G, Im HJ, You JS, Oh JS, Kim SG: Licochalcone A induces apoptosis in KB human oral cancer cells via a caspase-dependent FasL signaling pathway. *Oncol Rep* 2014;31:755-762.
- 8 Hao H, Hui W, Liu P, Lv Q, Zeng X, Jiang H, Wang Y, Zheng X, Zheng Y, Li J, Zhou X, Jiang Y: Effect of licochalcone A on growth and properties of *Streptococcus suis*. *PLoS One* 2013;8:e67728.
- 9 Shen H, Zeng G, Tang G, Cai X, Bi L, Huang C, Yang Y: Antimetastatic effects of licochalcone A on oral cancer via regulating metastasis-associated proteases. *Tumour Biol* 2014;35:7467-7474.
- 10 Shang F, Ming L, Zhou Z, Yu Y, Sun J, Ding Y, Jin Y: The effect of licochalcone A on cell-aggregates ECM secretion and osteogenic differentiation during bone formation in metaphyseal defects in ovariectomized rats. *Biomaterials* 2014;35:2789-2797.
- 11 Yao K, Chen H, Lee MH, Li H, Ma W, Peng C, Song NR, Lee KW, Bode AM, Dong Z, Dong Z: Licochalcone A, a natural inhibitor of c-Jun N-terminal kinase 1. *Cancer Prev Res (Phila)* 2014;7:139-149.

- 12 Tsai JP, Lee CH, Ying TH, Lin CL, Lin CL, Hsueh JT, Hsieh YH: Licochalcone A induces autophagy through PI3K/Akt/mTOR inactivation and autophagy suppression enhances Licochalcone A-induced apoptosis of human cervical cancer cells. *Oncotarget* 2015;
- 13 Cho JJ, Chae JI, Yoon G, Kim KH, Cho JH, Cho SS, Cho YS, Shim JH: Licochalcone A, a natural chalconoid isolated from *Glycyrrhiza inflata* root, induces apoptosis via Sp1 and Sp1 regulatory proteins in oral squamous cell carcinoma. *Int J Oncol* 2014;45:667-674.
- 14 Zeng G, Shen H, Yang Y, Cai X, Xun W: Licochalcone A as a potent antitumor agent suppresses growth of human oral cancer SCC-25 cells in vitro via caspase-3 dependent pathways. *Tumour Biol* 2014;35:6549-6555.
- 15 Park MR, Kim SG, Cho IA, Oh D, Kang KR, Lee SY, Moon SM, Cho SS, Yoon G, Kim CS, Oh JS, You JS, Kim do K, Seo YS, Im HJ, Kim JS: Licochalcone-A induces intrinsic and extrinsic apoptosis via ERK1/2 and p38 phosphorylation-mediated TRAIL expression in head and neck squamous carcinoma FaDu cells. *Food Chem Toxicol* 2015;77:34-43.
- 16 Yang P, Tuo L, Wu Q, Cao X: Licochalcone-A sensitizes human esophageal carcinoma cells to TRAIL-mediated apoptosis by proteasomal degradation of XIAP. *Hepatogastroenterology* 2014;61:1229-1234.
- 17 Hao W, Yuan X, Yu L, Gao C, Sun X, Wang D, Zheng Q: Licochalcone A-induced human gastric cancer BGC-823 cells apoptosis by regulating ROS-mediated MAPKs and PI3K/AKT signaling pathways. *Sci Rep* 2015;5:10336.
- 18 Xiao XY, Hao M, Yang XY, Ba Q, Li M, Ni SJ, Wang LS, Du X: Licochalcone A inhibits growth of gastric cancer cells by arresting cell cycle progression and inducing apoptosis. *Cancer Lett* 2011;302:69-75.
- 19 Choi AY, Choi JH, Hwang KY, Jeong YJ, Choe W, Yoon KS, Ha J, Kim SS, Youn JH, Yeo EJ, Kang I: Licochalcone A induces apoptosis through endoplasmic reticulum stress via a phospholipase Cgamma1-, Ca(2+)-, and reactive oxygen species-dependent pathway in HepG2 human hepatocellular carcinoma cells. *Apoptosis* 2014;19:682-697.
- 20 Kim KH, Yoon G, Cho JJ, Cho JH, Cho YS, Chae JI, Shim JH: Licochalcone A induces apoptosis in malignant pleural mesothelioma through downregulation of Sp1 and subsequent activation of mitochondria-related apoptotic pathway. *Int J Oncol* 2015;46:1385-1392.
- 21 Jiang J, Yuan X, Zhao H, Yan X, Sun X, Zheng Q: Licochalcone A inhibiting proliferation of bladder cancer T24 cells by inducing reactive oxygen species production. *Biomed Mater Eng* 2014;24:1019-1025.
- 22 Yuan X, Li D, Zhao H, Jiang J, Wang P, Ma X, Sun X, Zheng Q: Licochalcone A-induced human bladder cancer T24 cells apoptosis triggered by mitochondria dysfunction and endoplasmic reticulum stress. *Biomed Res Int* 2013;2013:474272.
- 23 Fu Y, Hsieh TC, Guo J, Kunicki J, Lee MY, Darzynkiewicz Z, Wu JM: Licochalcone-A, a novel flavonoid isolated from licorice root (*Glycyrrhiza glabra*), causes G2 and late-G1 arrests in androgen-independent PC-3 prostate cancer cells. *Biochem Biophys Res Commun* 2004;322:263-270.
- 24 Rafi MM, Rosen RT, Vassil A, Ho CT, Zhang H, Ghai G, Lambert G, DiPaola RS: Modulation of bcl-2 and cytotoxicity by licochalcone-A, a novel estrogenic flavonoid. *Anticancer Res* 2000;20:2653-2658.
- 25 Yo YT, Shieh GS, Hsu KF, Wu CL, Shiau AL: Licorice and licochalcone-A induce autophagy in LNCaP prostate cancer cells by suppression of Bcl-2 expression and the mTOR pathway. *J Agric Food Chem* 2009;57:8266-8273.
- 26 Kim YJ, Jung EB, Myung SC, Kim W, Lee CS: Licochalcone A enhances geldanamycin-induced apoptosis through reactive oxygen species-mediated caspase activation. *Pharmacology* 2013;92:49-59.
- 27 Lee CS, Kwak SW, Kim YJ, Lee SA, Park ES, Myung SC, Kim W, Lee MS, Lee JJ: Guanylate cyclase activator YC-1 potentiates apoptotic effect of licochalcone A on human epithelial ovarian carcinoma cells via activation of death receptor and mitochondrial pathways. *Eur J Pharmacol* 2012;683:54-62.
- 28 Huang HC, Tsai LL, Tsai JP, Hsieh SC, Yang SF, Hsueh JT, Hsieh YH: Licochalcone A inhibits the migration and invasion of human lung cancer cells via inactivation of the Akt signaling pathway with downregulation of MMP-1/-3 expression. *Tumour Biol* 2014;35:12139-12149.
- 29 Funakoshi-Tago M, Tago K, Nishizawa C, Takahashi K, Mashino T, Iwata S, Inoue H, Sonoda Y, Kasahara T: Licochalcone A is a potent inhibitor of TEL-Jak2-mediated transformation through the specific inhibition of Stat3 activation. *Biochem Pharmacol* 2008;76:1681-1693.
- 30 Park SY, Kim EJ, Choi HJ, Seon MR, Lim SS, Kang YH, Choi MS, Lee KW, Yoon Park JH: Anti-carcinogenic effects of non-polar components containing licochalcone A in roasted licorice root. *Nutr Res Pract* 2014;8:257-266.

- 31 Song NR, Kim JE, Park JS, Kim JR, Kang H, Lee E, Kang YG, Son JE, Seo SG, Heo YS, Lee KW: Licochalcone A, a polyphenol present in licorice, suppresses UV-induced COX-2 expression by targeting PI3K, MEK1, and B-Raf. *Int J Mol Sci* 2015;16:4453-4470.
- 32 He W, Wu JJ, Ning J, Hou J, Xin H, He YQ, Ge GB, Xu W: Inhibition of human cytochrome P450 enzymes by licochalcone A, a naturally occurring constituent of licorice. *Toxicol In Vitro* 2015;29:1569-1576.
- 33 Kuhl J, Roggenkamp D, Gehrke SA, Stab F, Wenck H, Kolbe L, Neufang G: Licochalcone A activates Nrf2 in vitro and contributes to licorice extract-induced lowered cutaneous oxidative stress in vivo. *Exp Dermatol* 2015;24:42-47.
- 34 Han J, Wang D, Yu B, Wang Y, Ren H, Zhang B, Wang Y, Zheng Q: Cardioprotection against ischemia/reperfusion by licochalcone B in isolated rat hearts. *Oxid Med Cell Longev* 2014;2014:134862.
- 35 Yuan X, Niu HT, Wang PL, Lu J, Zhao H, Liu SH, Zheng QS, Li CG: Cardioprotective Effect of Licochalcone D against Myocardial Ischemia/Reperfusion Injury in Langendorff-Perfused Rat Hearts. *PLoS One* 2015;10:e0128375.
- 36 Zhou M, Liu L, Wang W, Han J, Ren H, Zheng Q, Wang D: Role of licochalcone C in cardioprotection against ischemia/reperfusion injury of isolated rat heart via antioxidant, anti-inflammatory, and anti-apoptotic activities. *Life Sci* 2015;132:27-33.
- 37 Han JY, Park SH, Yang JH, Kim MG, Cho SS, Yoon G, Cheon SH, Ki SH: Licochalcone Suppresses LXRalpha-Induced Hepatic Lipogenic Gene Expression through AMPK/Sirt1 Pathway Activation. *Toxicol Res* 2014;30:19-25.
- 38 Wananukul S, Chatproedprai S, Chunharas A, Limpongsanuruk W, Singalavanija S, Nitiyaron R, Wisuthsarewong W: Randomized, double-blind, split-side, comparison study of moisturizer containing licochalcone A and 1% hydrocortisone in the treatment of childhood atopic dermatitis. *J Med Assoc Thai* 2013;96:1135-1142.
- 39 Chu X, Jiang L, Wei M, Yang X, Guan M, Xie X, Wei J, Liu D, Wang D: Attenuation of allergic airway inflammation in a murine model of asthma by Licochalcone A. *Immunopharmacol Immunotoxicol* 2013;35:653-661.
- 40 Kim SH, Yang M, Xu JG, Yu X, Qian XJ: Role of licochalcone A on thymic stromal lymphopoietin expression: implications for asthma. *Exp Biol Med (Maywood)* 2015;240:26-33.
- 41 Ming L, Jin F, Huang P, Luo H, Liu W, Zhang L, Yuan W, Zhang Y, Jin Y: Licochalcone A up-regulates of FasL in mesenchymal stem cells to strengthen bone formation and increase bone mass. *Sci Rep* 2014;4:7209.
- 42 Oh GS, Lee GG, Yoon J, Oh WK, Kim SW: Selective inhibition of liver X receptor alpha-mediated lipogenesis in primary hepatocytes by licochalcone A. *Chin Med* 2015;10:8.
- 43 Quan HY, Baek NI, Chung SH: Licochalcone A prevents adipocyte differentiation and lipogenesis via suppression of peroxisome proliferator-activated receptor gamma and sterol regulatory element-binding protein pathways. *J Agric Food Chem* 2012;60:5112-5120.
- 44 Quan HY, Kim SJ, Kim do Y, Jo HK, Kim GW, Chung SH: Licochalcone A regulates hepatic lipid metabolism through activation of AMP-activated protein kinase. *Fitoterapia* 2013;86:208-216.
- 45 Bak EJ, Choi KC, Jang S, Woo GH, Yoon HG, Na Y, Yoo YJ, Lee Y, Jeong Y, Cha JH: Licochalcone F alleviates glucose tolerance and chronic inflammation in diet-induced obese mice through Akt and p38 MAPK. *Clin Nutr* 2015;10.1016/j.clnu.2015.03.005
- 46 Yuan X, Li T, Xiao E, Zhao H, Li Y, Fu S, Gan L, Wang Z, Zheng Q, Wang Z: Licochalcone B inhibits growth of bladder cancer cells by arresting cell cycle progression and inducing apoptosis. *Food Chem Toxicol* 2014;65:242-251.
- 47 Zhao H, Yuan X, Jiang J, Wang P, Sun X, Wang D, Zheng Q: Antimetastatic effects of licochalcone B on human bladder carcinoma T24 by inhibition of matrix metalloproteinases-9 and NF-small ka, CyrillicB activity. *Basic Clin Pharmacol Toxicol* 2014;115:527-533.
- 48 Chang HJ, Yoon G, Park JS, Kim MH, Baek MK, Kim NH, Shin BA, Ahn BW, Cheon SH, Jung YD: Induction of apoptosis by the licochalcone E in endothelial cells via modulation of NF-kappaB and Bcl-2 Family. *Biol Pharm Bull* 2007;30:2290-2293.
- 49 Kwon SJ, Park SY, Kwon GT, Lee KW, Kang YH, Choi MS, Yun JW, Jeon JH, Jun JG, Park JH: Licochalcone E present in licorice suppresses lung metastasis in the 4T1 mammary orthotopic cancer model. *Cancer Prev Res (Phila)* 2013;6:603-613.
- 50 Zhou T, Deng X, Qiu J: Antimicrobial activity of licochalcone E against *Staphylococcus aureus* and its impact on the production of staphylococcal alpha-toxin. *J Microbiol Biotechnol* 2012;22:800-805.

- 51 Lang E, Qadri SM, Lang F: Killing me softly - suicidal erythrocyte death. *Int J Biochem Cell Biol* 2012;44:1236-1243.
- 52 Lang PA, Kaiser S, Myssina S, Wieder T, Lang F, Huber SM: Role of Ca²⁺-activated K⁺ channels in human erythrocyte apoptosis. *Am J Physiol Cell Physiol* 2003;285:C1553-C1560.
- 53 Harrison HE, Bunting H, Ordway NK, Albrink WS: The Pathogenesis of the Renal Injury Produced in the Dog by Hemoglobin or Methemoglobin. *J Exp Med* 1947;86:339-356.
- 54 Borst O, Abed M, Alesutan I, Towhid ST, Qadri SM, Foller M, Gawaz M, Lang F: Dynamic adhesion of eryptotic erythrocytes to endothelial cells via CXCL16/SR-PSOX. *Am J Physiol Cell Physiol* 2012;302:C644-C651.
- 55 Andrews DA, Low PS: Role of red blood cells in thrombosis. *Curr Opin Hematol* 1999;6:76-82.
- 56 Chung SM, Bae ON, Lim KM, Noh JY, Lee MY, Jung YS, Chung JH: Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant microvesicle generation in human erythrocytes. *Arterioscler Thromb Vasc Biol* 2007;27:414-421.
- 57 Zwaal RF, Comfurius P, Bevers EM: Surface exposure of phosphatidylserine in pathological cells. *Cell Mol Life Sci* 2005;62:971-988.
- 58 Abed M, Towhid ST, Mia S, Pakladok T, Alesutan I, Borst O, Gawaz M, Gulbins E, Lang F: Sphingomyelinase-induced adhesion of eryptotic erythrocytes to endothelial cells. *Am J Physiol Cell Physiol* 2012;303:C991-999.
- 59 Closse C, Dachary-Prigent J, Boisseau MR: Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. *Br J Haematol* 1999;107:300-302.
- 60 Gallagher PG, Chang SH, Rettig MP, Neely JE, Hillery CA, Smith BD, Low PS: Altered erythrocyte endothelial adherence and membrane phospholipid asymmetry in hereditary hydrocytosis. *Blood* 2003;101:4625-4627.
- 61 Pandolfi A, Di Pietro N, Sirolli V, Giardinelli A, Di Silvestre S, Amoroso L, Di Tomo P, Capani F, Consoli A, Bonomini M: Mechanisms of uremic erythrocyte-induced adhesion of human monocytes to cultured endothelial cells. *J Cell Physiol* 2007;213:699-709.
- 62 Wood BL, Gibson DF, Tait JF: Increased erythrocyte phosphatidylserine exposure in sickle cell disease: flow-cytometric measurement and clinical associations. *Blood* 1996;88:1873-1880.
- 63 Abed M, Feger M, Alzoubi K, Pakladok T, Frauenfeld L, Geiger C, Towhid ST, Lang F: Sensitization of erythrocytes to suicidal erythrocyte death following water deprivation. *Kidney Blood Press Res* 2013;37:567-578.
- 64 Voelkl J, Alzoubi K, Mamar AK, Ahmed MS, Abed M, Lang F: Stimulation of suicidal erythrocyte death by increased extracellular phosphate concentrations. *Kidney Blood Press Res* 2013;38:42-51.
- 65 Abed M, Artunc F, Alzoubi K, Honisch S, Baumann D, Foller M, Lang F: Suicidal erythrocyte death in end-stage renal disease. *J Mol Med (Berl)* 2014;92:871-879.
- 66 Ahmed MS, Langer H, Abed M, Voelkl J, Lang F: The uremic toxin acrolein promotes suicidal erythrocyte death. *Kidney Blood Press Res* 2013;37:158-167.
- 67 Polak-Jonkisz D, Purzyc L: Ca²⁺ influx versus efflux during eryptosis in uremic erythrocytes. *Blood Purif* 2012;34:209-210; author reply 210.
- 68 Calderon-Salinas JV, Munoz-Reyes EG, Guerrero-Romero JF, Rodriguez-Moran M, Bracho-Riquelme RL, Carrera-Gracia MA, Quintanar-Escorza MA: Eryptosis and oxidative damage in type 2 diabetic mellitus patients with chronic kidney disease. *Mol Cell Biochem* 2011;357:171-179.
- 69 Lang PA, Beringer O, Nicolay JP, Amon O, Kempe DS, Hermle T, Attanasio P, Akel A, Schafer R, Friedrich B, Risler T, Baur M, Olbricht CJ, Zimmerhackl LB, Zipfel PF, Wieder T, Lang F: Suicidal death of erythrocytes in recurrent hemolytic uremic syndrome. *J Mol Med (Berl)* 2006;84:378-388.
- 70 Nicolay JP, Schneider J, Niemoeller OM, Artunc F, Portero-Otin M, Haik G, Jr., Thornalley PJ, Schleicher E, Wieder T, Lang F: Stimulation of suicidal erythrocyte death by methylglyoxal. *Cell Physiol Biochem* 2006;18:223-232.
- 71 Lang E, Gatidis S, Freise NF, Bock H, Kubitz R, Lauermann C, Orth HM, Klindt C, Schuier M, Keitel V, Reich M, Liu G, Schmidt S, Xu HC, Qadri SM, Herebian D, Pandya AA, Mayatepek E, Gulbins E, Lang F, Haussinger D, Lang KS, Foller M, Lang PA: Conjugated bilirubin triggers anemia by inducing erythrocyte death. *Hepatology* 2015;61:275-284.
- 72 Kempe DS, Akel A, Lang PA, Hermle T, Biswas R, Muresanu J, Friedrich B, Dreischer P, Wolz C, Schumacher U, Peschel A, Gotz F, Doring G, Wieder T, Gulbins E, Lang F: Suicidal erythrocyte death in sepsis. *J Mol Med (Berl)* 2007;85:273-281.

- 73 Lang PA, Schenck M, Nicolay JP, Becker JU, Kempe DS, Lupescu A, Koka S, Eisele K, Klarl BA, Rubben H, Schmid KW, Mann K, Hildenbrand S, Hefter H, Huber SM, Wieder T, Erhardt A, Haussinger D, Gulbins E, Lang F: Liver cell death and anemia in Wilson disease involve acid sphingomyelinase and ceramide. *Nat Med* 2007;13:164-170.
- 74 Jilani K, Lang F: Carmustine-induced phosphatidylserine translocation in the erythrocyte membrane. *Toxins (Basel)* 2013;5:703-716.
- 75 Vota DM, Maltaner RE, Wenker SD, Nesse AB, Vittori DC: Differential erythropoietin action upon cells induced to eryptosis by different agents. *Cell Biochem Biophys* 2013;65:145-157.
- 76 Zappulla D: Environmental stress, erythrocyte dysfunctions, inflammation, and the metabolic syndrome: adaptations to CO2 increases? *J Cardiometa Syndr* 2008;3:30-34.
- 77 Lupescu A, Jilani K, Zbidah M, Lang F: Patulin-induced suicidal erythrocyte death. *Cell Physiol Biochem* 2013;32:291-299.
- 78 Abed M, Zoubi KA, Theurer M, Lang F: Effect of dermaseptin on erythrocytes. *Basic Clin Pharmacol Toxicol* 2013;113:347-352.
- 79 Ghashghaieina M, Cluitmans JC, Toulany M, Saki M, Koberle M, Lang E, Dreischer P, Biedermann T, Duzenko M, Lang F, Bosman GJ, Wieder T: Age Sensitivity of NFkappaB Abundance and Programmed Cell Death in Erythrocytes Induced by NFkappaB Inhibitors. *Cell Physiol Biochem* 2013;32:801-813.
- 80 Alzoubi K, Honisch S, Abed M, Lang F: Triggering of Suicidal Erythrocyte Death by Penta-O-galloyl-beta-d-glucose. *Toxins (Basel)* 2014;6:54-65.
- 81 Jilani K, Enkel S, Bissinger R, Almilaji A, Abed M, Lang F: Fluoxetine induced suicidal erythrocyte death. *Toxins (Basel)* 2013;5:1230-1243.
- 82 Lupescu A, Bissinger R, Jilani K, Lang F: Triggering of suicidal erythrocyte death by celecoxib. *Toxins (Basel)* 2013;5:1543-1554.
- 83 Arnold M, Lang E, Modicano P, Bissinger R, Faggio C, Abed M, Lang F: Effect of nitazoxanide on erythrocytes. *Basic Clin Pharmacol Toxicol* 2014;114:421-426.
- 84 Oswald G, Alzoubi K, Abed M, Lang F: Stimulation of suicidal erythrocyte death by ribavirin. *Basic Clin Pharmacol Toxicol* 2014;114:311-317.
- 85 Lupescu A, Bissinger R, Herrmann T, Oswald G, Jilani K, Lang F: Induction of suicidal erythrocyte death by novobiocin. *Cell Physiol Biochem* 2014;33:670-680.
- 86 Alzoubi K, Calabrò S, Bissinger R, Abed M, Faggio C, Lang F: Stimulation of Suicidal Erythrocyte Death by Artesunate. *Cell Physiol Biochem* 2014;34:2232-2244.
- 87 Arnold M, Bissinger R, Lang F: Mitoxantrone-induced suicidal erythrocyte death. *Cell Physiol Biochem* 2014;34:1756-1767.
- 88 Bissinger R, Fischer S, Jilani K, Lang F: Stimulation of Erythrocyte Death by Phloretin. *Cell Physiol Biochem* 2014;34:2256-2265.
- 89 Bissinger R, Lupescu A, Zelenak C, Jilani K, Lang F: Stimulation of eryptosis by cryptotanshinone. *Cell Physiol Biochem* 2014;34:432-442.
- 90 Bissinger R, Modicano P, Frauenfeld L, Lang E, Jacobi J, Faggio C, Lang F: Estramustine-induced suicidal erythrocyte death. *Cell Physiol Biochem* 2013;32:1426-1436.
- 91 Jacobi J, Lang E, Bissinger R, Frauenfeld L, Modicano P, Faggio C, Abed M, Lang F: Stimulation of erythrocyte cell membrane scrambling by mitotane. *Cell Physiol Biochem* 2014;33:1516-1526.
- 92 Lupescu A, Bissinger R, Warsi J, Jilani K, Lang F: Stimulation of erythrocyte cell membrane scrambling by gedunin. *Cell Physiol Biochem* 2014;33:1838-1848.
- 93 Malik A, Bissinger R, Calabro S, Faggio C, Jilani K, Lang F: Aristolochic Acid Induced Suicidal Erythrocyte Death. *Kidney Blood Press Res* 2014;39:408-419.
- 94 Tesoriere L, Attanzio A, Allegra M, Cilla A, Gentile C, Livrea MA: Oxysterol mixture in hypercholesterolemia-relevant proportion causes oxidative stress-dependent eryptosis. *Cell Physiol Biochem* 2014;34:1075-1089.
- 95 Zhang R, Xiang Y, Ran Q, Deng X, Xiao Y, Xiang L, Li Z: Involvement of calcium, reactive oxygen species, and ATP in hexavalent chromium-induced damage in red blood cells. *Cell Physiol Biochem* 2014;34:1780-1791.
- 96 Pagano M, Faggio C: The use of erythrocyte fragility to assess xenobiotic cytotoxicity. *Cell Biochem Funct* 2015;33:351-355.