Original Paper

Triggering of Eryptosis, the Suicidal Erythrocyte Death, by Perifosine

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Key Words

Phosphatidylserine • Eryptosis • Staurosporine • Ceramide • Oxidative stress • Calcium

Abstract

Background/Aims: The alkylphospholipid perifosine is used for the treatment of malignancy. The substance is effective by triggering suicidal tumor cell death or apoptosis. Side effects of perifosine include anemia. At least in theory, perifosine-induced anemia could result from stimulation of suicidal erythrocyte death or eryptosis. Hallmarks of eryptosis are cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Cellular mechanisms participating in the orchestration of eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), oxidative stress, increase of ceramide abundance, as well as activation of staurosporine sensitive protein kinase C and/or of SB203580 sensitive p38 kinase. The present study explored, whether perifosine induces eryptosis and, if so, whether its effect involves and/or requires Ca^{2+} entry, oxidative stress, ceramide and kinase activation.

Methods: Flow cytometry was employed to quantify phosphatidylserine exposure at the cell surface from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, reactive oxygen species (ROS) abundance from DCFDA dependent fluorescence, and ceramide abundance utilizing specific antibodies. Hemolysis was estimated from hemoglobin concentration in the supernatant. **Results:** A 24 hours exposure of human erythrocytes to perifosine (2.5 $\mu\text{g}/\text{ml}$) significantly increased the percentage of annexin-V-binding cells, significantly decreased average forward scatter, significantly increased the percentage of shrunken erythrocytes, and significantly decreased the percentage of swollen erythrocytes. Perifosine significantly increased the percentage of hemolytic erythrocytes. Perifosine significantly increased Fluo3-fluorescence, but decreased DCFDA fluorescence and ceramide abundance. The effect of perifosine on annexin-V-binding was significantly blunted by removal of extracellular Ca^{2+} and by addition of staurosporine (1 μM), but not by addition of SB203580 (2 μM). **Conclusions:** Perifosine triggers eryptosis, an effect at least in part due to Ca^{2+} entry and activation of staurosporine sensitive kinases.

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Introduction

Perifosine, an alkylphospholipid drug [1, 2], is effective against malignancy [1-19]. Mechanisms involved in the action of perifosine include interference with the activation of

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Akt [1-4, 6, 8-13, 15, 16, 18, 20, 21] and stimulation of death receptor clustering [3, 22] with subsequent triggering of suicidal cell death or apoptosis [3, 8, 11-14, 16-18, 21, 23]. On the other hand, perifosine could activate AMP activated kinase AMPK and protect against cell death [24].

At least in theory, the anemia could result from suicidal death of erythrocytes or eryptosis [25]. Eryptosis is characterized by cell membrane scrambling with phosphatidylserine translocation to the cell surface [25]. A second hallmark of eryptosis is cell shrinkage [26]. Cellular mechanisms involved in the execution of eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [25], ceramide [27], caspases [25, 28, 29], casein kinase 1 α [25], Janus-activated kinase JAK3 [25], protein kinase C [25], and p38 kinase [25]. Eryptosis is inhibited by PAK2 kinase [25], AMP activated kinase AMPK [25], cGMP-dependent protein kinase [25], and sorafenib/sunitinib sensitive kinases [25]. Triggers of eryptosis include hyperosmotic shock [25], oxidative stress [25], energy depletion [25], and a myriad of xenobiotics [25, 30-81].

The present study explored, whether perifosine may stimulate eryptosis. To this end, human erythrocytes from healthy volunteers were treated with perifosine and phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$, reactive oxygen species (ROS) formation, and ceramide abundance determined by flow cytometry.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Li-Heparin-anticoagulated blood samples were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003 V). The blood was centrifuged at 120 g for 20 min at 21 °C and the platelets and leukocytes-containing supernatant was disposed. Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, 1 CaCl_2 , at 37°C for 24 hours. Where indicated, erythrocytes were exposed for 24 hours to perifosine (Sigma, Schnelldorf, Germany). In order to estimate the impact of Ca^{2+} entry on perifosine induced eryptosis, erythrocytes were exposed to perifosine in the absence of extracellular Ca^{2+} and presence of Ca^{2+} chelator EGTA (1 mM, Merck Millipore, Darmstadt, Germany). To test for an involvement of kinases, erythrocytes were exposed for 24 hours to a combination of perifosine and p38 kinase inhibitor SB203580 (Tocris bioscience, Bristol, UK) or protein kinase C inhibitor staurosporine (Enzo Life Sciences, Lörrach, Germany).

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 100 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. The annexin-V-abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). Annexin-V-binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin-V-binding cells and control cells. The same threshold was used for untreated and perifosine treated erythrocytes. A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters. The threshold of forward scatter was set at the default value of "52".

Intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and 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. 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. Afterwards, the geomean of the Ca^{2+} dependent fluorescence was determined.

Reactive oxidant species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 200 μ l suspension of erythrocytes was washed in Ringer solution and stained with DCFDA (Sigma, Schnelldorf, Germany) in Ringer solution containing DCFDA at a final concentration of 10 μ M. Erythrocytes were incubated at 37°C for 30 min in the dark and washed two times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 μ l Ringer solution and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD). Subsequently, the geomean of the DCFDA dependent fluorescence was determined.

Ceramide abundance

For the determination of ceramide, a monoclonal antibody-based assay was used. To this end, cells were stained for 1 hour at 37°C with 1 μ g/ml anti ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. The samples were washed twice with PBS-BSA. The cells were stained for 30 minutes with polyclonal fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. The samples were analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Finally, the geomean of the ceramide-dependent fluorescence was determined.

Hemolysis

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

Statistics

Data are expressed as arithmetic means $\pm$ 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, whether the alkylphospholipid drug perifosine stimulates eryptosis, the suicidal erythrocyte death. Hallmarks of eryptosis are phospholipid scrambling of the cell membrane and erythrocyte shrinkage.

Cell membrane scrambling was apparent from phosphatidylserine translocation to the erythrocyte surface which was visualized utilizing annexin-V-binding to phosphatidylserine, as determined by flow cytometry. Prior to determination of annexin-V-binding, the erythrocytes were incubated for 24 hours in Ringer solution without or with perifosine (0.25 – 2.5 μ g/ml). As illustrated in Fig. 1, a 24 hours exposure to perifosine increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 2.5 μ g/ml perifosine.

Erythrocyte shrinkage was evidenced by measuring erythrocyte forward scatter utilizing flow cytometry. The measurements were performed after incubation for 24 hours in Ringer solution without or with perifosine (0.25 – 2.5 μ g/ml). As a result, exposure to perifosine decreased the average erythrocyte forward scatter, an effect reaching statistical significance at 2.5 μ g/ml perifosine (Fig. 2A,B). Moreover, perifosine increased the percentage of shrunken erythrocytes (Fig. 2C) and decreased the percentage of swollen erythrocytes (Fig. 2D), effects reaching again statistical significance at 2.5 μ g/ml perifosine. The observations revealed that perifosine did induce eryptosis.

Fig. 1. Effect of perifosine 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 2.5 $\mu\text{g/ml}$ perifosine. B. Arithmetic means $\pm$ SEM (n = 16) of erythrocyte annexin-V-binding following incubation for 24 hours to Ringer solution without (white bar) or with (black bars) perifosine (0.25 – 2.5 $\mu\text{g/ml}$). ***($p < 0.001$) indicates significant difference from the absence of perifosine (ANOVA).

Fig. 2. Effect of perifosine on erythrocyte forward scatter. A. Original histograms of forward scatter of erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of 2.5 $\mu\text{g/ml}$ perifosine. B. Arithmetic means $\pm$ SEM (n = 16) of the erythrocyte forward scatter (FSC) following incubation for 24 hours to Ringer solution without (white bar) or with (black bars) perifosine (0.25 – 2.5 $\mu\text{g/ml}$). C,D. Percentage of erythrocytes with (C) FSC < 200 or (D) FSC > 800 following incubation for 24 hours to Ringer solution without (white bar) or with (black bars) perifosine (0.25 – 2.5 $\mu\text{g/ml}$). *($p < 0.05$), ***($p < 0.001$) indicate significant difference from the absence of perifosine (ANOVA).

Fig. 3. Effect of perifosine on hemolysis. Arithmetic means $\pm$ SEM (n = 8) of the percentage of hemolysed erythrocytes following incubation for 24 hours to Ringer solution without (white bar) or with (black bars) perifosine (0.25 – 2.5 $\mu\text{g/ml}$). *($p < 0.05$), ***($p < 0.001$) indicates significant difference from the absence of perifosine (ANOVA).

The stimulation of eryptosis was paralleled by stimulation of hemolysis. As illustrated in Fig. 3, a 24 hours exposure to perifosine increased the percentage of hemolytic erythrocytes, an effect reaching statistical significance at 0.5 $\mu\text{g/ml}$ perifosine.

Eryptosis could be triggered by increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Fluo3 fluorescence was employed in order to quantify $[\text{Ca}^{2+}]_i$. The erythrocytes were analyzed after a 24 hours incubation in Ringer solution without or with perifosine (0.25 – 2.5 $\mu\text{g/ml}$). As illustrated in Fig. 4, a 24 hours exposure to perifosine increased the Fluo3 fluorescence, an effect reaching statistical significance at 2.5 $\mu\text{g/ml}$ perifosine.

Fig. 4. Effect of perifosine on cytosolic Ca^{2+} activity. A. Original histogram of Fluo3 fluorescence of erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of 2.5 $\mu\text{g/ml}$ perifosine. B. Arithmetic means $\pm$ SEM ($n = 12$) of erythrocyte Fluo3 fluorescence following incubation for 24 hours to Ringer solution without (white bar) or with (black bars) perifosine (0.25 – 2.5 $\mu\text{g/ml}$). **($p < 0.01$) indicates significant difference from the absence of perifosine (ANOVA).

Fig. 4. Effect of perifosine on cytosolic Ca^{2+} activity. A. Original histogram of Fluo3 fluorescence of erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of 2.5 $\mu\text{g/ml}$ perifosine. B. Arithmetic means $\pm$ SEM ($n = 12$) of erythrocyte Fluo3 fluorescence following incubation for 24 hours to Ringer solution without (white bar) or with (black bars) perifosine (0.25 – 2.5 $\mu\text{g/ml}$). **($p < 0.01$) indicates significant difference from the absence of perifosine (ANOVA).

Fig. 5. Ca^{2+} sensitivity of perifosine-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 24 hours to Ringer solution without (grey areas) and with (black lines) perifosine (2.5 $\mu\text{g/ml}$) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means $\pm$ SEM ($n = 12$) of annexin-V-binding of erythrocytes after a 24 hours treatment with Ringer solution without (white bars) or with (black bars) perifosine (2.5 $\mu\text{g/ml}$) in the presence (left bars, + Ca^{2+}) and absence (right bars, - Ca^{2+}) of Ca^{2+} . ***($p < 0.001$) indicates significant difference from the absence of perifosine, ##($p < 0.01$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

In order to test, whether perifosine-induced translocation of phosphatidylserine to the outside required entry of extracellular Ca^{2+} , erythrocytes were incubated for 24 hours in the absence or presence of 2.5 $\mu\text{g/ml}$ perifosine in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 5, removal of extracellular Ca^{2+} significantly blunted the effect of perifosine on annexin-V-binding. However, even in the absence of extracellular Ca^{2+} , perifosine significantly increased the percentage of annexin-V-binding erythrocytes. Thus, the perifosine-induced cell membrane scrambling was in part but not fully triggered by entry of extracellular Ca^{2+} .

To explore, whether the effects of perifosine involved kinase activity, the influence of perifosine on annexin-V-binding was tested in the presence of p38 kinase inhibitor SB203580, or of protein kinase C inhibitor staurosporine. As illustrated in Fig. 6, staurosporine (1 μM) significantly blunted the effect of perifosine on annexin-V-binding. However, even in the presence of staurosporine, perifosine significantly increased the percentage of annexin-V-binding erythrocytes. Accordingly, perifosine-induced cell membrane scrambling was in part but not fully dependent on staurosporine sensitive kinases. As further illustrated in Fig. 6, SB203580 (2 μM) tended to blunt the perifosine-induced annexin-V-binding, an effect, however, not reaching statistical significance.

Eryptosis is further stimulated by oxidative stress [25]. Reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As illustrated in Fig. 7, a 24 hours exposure to perifosine (2.5 $\mu\text{g/ml}$) significantly decreased

Fig. 6. Staurosporine and SB203580 sensitivity of perifosine-induced phosphatidylserine exposure. A,B,C. Original histograms of annexin-V-binding of erythrocytes following exposure for 24 hours to Ringer solution without (grey areas) and with (black lines) perifosine (2.5 $\mu\text{g}/\text{ml}$) in the absence (A) and presence of 2 μM SB203580 (B) or 1 μM staurosporine (C). D. Arithmetic means $\pm$ SEM ($n = 8$) of annexin-V-binding of erythrocytes after a 24 hours treatment with Ringer solution without (white bars) or with (black bars) perifosine (2.5 $\mu\text{g}/\text{ml}$) in the absence (left bars) and presence of 2 μM SB203580 (middle bars) or 1 μM staurosporine (right bars). ***($p < 0.001$) indicates significant difference from the absence of perifosine, #($p < 0.05$) indicates significant difference from the absence of kinase inhibitors (ANOVA).

Fig. 7. Effect of perifosine on reactive oxygen species. A. Original histogram of DCFDA fluorescence in erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of 2.5 $\mu\text{g}/\text{ml}$ perifosine. B. Arithmetic means $\pm$ SEM ($n = 12$) of DCFDA fluorescence in erythrocytes following incubation for 24 hours to Ringer solution without (white bar) or with (black bar) perifosine (2.5 $\mu\text{g}/\text{ml}$). **($p < 0.01$) indicates significant difference from the absence of perifosine (unpaired t test).

Fig. 8. Effect of perifosine on ceramide abundance. A. Original histogram of ceramide abundance in erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of 2.5 $\mu\text{g}/\text{ml}$ perifosine. B. Arithmetic means $\pm$ SEM ($n = 8$) of ceramide abundance in erythrocytes following incubation for 24 hours to Ringer solution without (white bar) or with (black bar) perifosine (2.5 $\mu\text{g}/\text{ml}$). **($p < 0.01$) indicates significant difference from the absence of perifosine (unpaired t test).

the DCFDA fluorescence of erythrocytes. Thus, perifosine rather decreased oxidative stress.

A further stimulator of eryptosis is ceramide [25]. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As shown in Fig. 8, a 24 hours exposure to perifosine (2.5 $\mu\text{g}/\text{ml}$) significantly decreased the ceramide abundance at the erythrocyte surface.

Discussion

The present observations disclose a novel effect of the cytostatic drug perifosine, i.e. the triggering of erythrocyte shrinkage and stimulation of cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Erythrocyte shrinkage and cell membrane scrambling are the hallmarks of eryptosis, the suicidal erythrocyte death [25]. The perifosine concentrations required for the stimulation of eryptosis are slightly higher than the concentrations (0.6 µg/ml) reported in the plasma of patients under perifosine treatment [82]. It must be kept in mind that the experiments have been performed with erythrocytes from healthy individuals. The susceptibility to undergo eryptosis is enhanced in diverse clinical conditions, including iron deficiency [25], dehydration [83], hyperphosphatemia [84], chronic kidney disease (CKD) [85-88], hemolytic-uremic syndrome [89], diabetes [90], hepatic failure [30], malignancy [25], sepsis [91], fever [25], malaria [25], sickle-cell disease [25], beta-thalassemia [25], Hb-C and G6PD-deficiency [25], as well as Wilsons disease [91]. The stimulation of eryptosis may thus well explain the anemia following perifosine treatment.

The effect of perifosine on cell membrane scrambling was paralleled by an increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) and was significantly blunted by removal of extracellular Ca^{2+} . Thus, perifosine-induced eryptosis was apparently in part dependent on Ca^{2+} entry from the extracellular space. However, perifosine triggered cell membrane scrambling even in the nominal absence of extracellular Ca^{2+} , an observation suggesting the involvement of additional mechanisms contributing to perifosine induced cell membrane scrambling. The erythrocytes are sensitized to the scrambling effect of Ca^{2+} by ceramide [25]. However, perifosine rather decreased ceramide abundance. Perifosine further significantly decreased the abundance of reactive oxygen species, a well known stimulator of eryptosis [25]. Thus, perifosine was not effective by inducing oxidative stress. The effect of perifosine was not significantly blunted in the presence of p38 kinase blocker SB203580, but was significantly blunted in the presence of staurosporine. Thus, staurosporine sensitive kinases but not p38 kinase may be required for the full effect of perifosine on eryptosis. Both, SB203580 and staurosporine sensitive kinases are known to participate in the orchestration of eryptosis by other stimuli [25].

The effect of perifosine on erythrocyte shrinkage is presumably at least in part secondary to increase of $[\text{Ca}^{2+}]_i$ with subsequent activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [25].

Eryptotic erythrocytes are rapidly cleared from circulating blood by uptake into phagocytes [25]. The clearance of defective erythrocytes from circulating blood by eryptosis may precede hemolysis and thus accomplish clearance of defective erythrocytes prior to hemolysis [25]. Perifosine treatment is followed by hemolysis pointing to disruption of the cell membrane integrity. *In vitro*, the defective erythrocytes cannot be engulfed by phagocytes and thus not be removed. *In vivo*, hemolysis leads to release of hemoglobin which may pass the renal glomerular filter, precipitate in the acidic lumen of renal tubules, occlude nephrons and may thus lead to renal failure [92].

The clearance of defective erythrocytes following eryptosis or hemolysis is expected to trigger anemia as soon as the loss of erythrocytes surpasses the formation of new erythrocytes by erythropoiesis [25]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [93], stimulate blood clotting and trigger thrombosis [94-96], and may thus interfere with microcirculation [27, 94, 97-100].

In conclusion, perifosine triggers erythrocyte cell membrane scrambling, an effect involving Ca^{2+} entry and activation of staurosporine sensitive kinases.

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Disclosure Statement

The authors declare no conflict of interest.

References

- 1 Srivastava N, Cho DC: Perifosine in renal cell carcinoma. *Expert Opin Investig Drugs* 2013;22:285-291.
- 2 Shen J, Hong Y, Zhao Q, Zhang JL: Preclinical evaluation of perifosine as a potential promising anti-rhabdomyosarcoma agent. *Tumour Biol* 2016;37:1025-1033.
- 3 Fensterle J, Aicher B, Seipelt I, Teifel M, Engel J: Current view on the mechanism of action of perifosine in cancer. *Anticancer Agents Med Chem* 2014;14:629-635.
- 4 Krawczyk J, Keane N, Swords R, O'Dwyer M, Freeman CL, Giles FJ: Perifosine--a new option in treatment of acute myeloid leukemia? *Expert Opin Investig Drugs* 2013;22:1315-1327.
- 5 Dogan SS, Esmaeli B: Ocular side effects associated with imatinib mesylate and perifosine for gastrointestinal stromal tumor. *Hematol Oncol Clin North Am* 2009;23:109-114, ix.
- 6 Gills JJ, Dennis PA: Perifosine: update on a novel Akt inhibitor. *Curr Oncol Rep* 2009;11:102-110.
- 7 Conley AP, Araujo D, Ludwig J, Ravi V, Samuels BL, Choi H, Thall PF, Patel S, Benjamin R, Trent J: A randomized phase II study of perifosine (P) plus imatinib for patients with imatinib-resistant gastrointestinal stromal tumor (GIST). *J Clin Oncol* 2009;27:10563.
- 8 Shen J, Xu L, Zhao Q: Perifosine and ABT-737 synergistically inhibit lung cancer cells in vitro and in vivo. *Biochem Biophys Res Commun* 2016;473:1170-1176.
- 9 Amantini C, Morelli MB, Santoni M, Soriani A, Cardinali C, Farfariello V, Eleuteri AM, Bonfili L, Mozzicafreddo M, Nabissi M, Cascinu S, Santoni G: Sorafenib induces cathepsin B-mediated apoptosis of bladder cancer cells by regulating the Akt/PTEN pathway. The Akt inhibitor, perifosine, enhances the sorafenib-induced cytotoxicity against bladder cancer cells. *Oncoscience* 2015;2:395-409.
- 10 Kim MN, Ro SW, Kim DY, Kim da Y, Cho KJ, Park JH, Lim HY, Han KH: Efficacy of perifosine alone and in combination with sorafenib in an HrasG12V plus shp53 transgenic mouse model of hepatocellular carcinoma. *Cancer Chemother Pharmacol* 2015;76:257-267.
- 11 Zhang J, Hong Y, Shen J: Combination treatment with perifosine and MEK-162 demonstrates synergism against lung cancer cells in vitro and in vivo. *Tumour Biol* 2015;36:5699-5706.
- 12 Qin LS, Yu ZQ, Zhang SM, Sun G, Zhu J, Xu J, Guo J, Fu LS: The short chain cell-permeable ceramide (C6) restores cell apoptosis and perifosine sensitivity in cultured glioblastoma cells. *Mol Biol Rep* 2013;40:5645-5655.
- 13 Locatelli SL, Giacomini A, Guidetti A, Cleris L, Mortarini R, Anichini A, Gianni AM, Carlo-Stella C: Perifosine and sorafenib combination induces mitochondrial cell death and antitumor effects in NOD/SCID mice with Hodgkin lymphoma cell line xenografts. *Leukemia* 2013;27:1677-1687.
- 14 Yao C, Wei JJ, Wang ZY, Ding HM, Li D, Yan SC, Yang YJ, Gu ZP: Perifosine induces cell apoptosis in human osteosarcoma cells: new implication for osteosarcoma therapy? *Cell Biochem Biophys* 2013;65:217-227.
- 15 Block M, Grundker C, Fister S, Kubin J, Wilkens L, Mueller MD, Hemmerlein B, Emons G, Gunthert AR: Inhibition of the AKT/mTOR and erbB pathways by gefitinib, perifosine and analogs of gonadotropin-releasing hormone I and II to overcome tamoxifen resistance in breast cancer cells. *Int J Oncol* 2012;41:1845-1854.
- 16 Gills JJ, Zhang C, Abu-Asab MS, Castillo SS, Marceau C, LoPiccolo J, Kozikowski AP, Tsokos M, Goldkorn T, Dennis PA: Ceramide mediates nanovesicle shedding and cell death in response to phosphatidylinositol ether lipid analogs and perifosine. *Cell Death Dis* 2012;3:e340.
- 17 Chen MB, Wu XY, Tao GQ, Liu CY, Chen J, Wang LQ, Lu PH: Perifosine sensitizes curcumin-induced anti-colorectal cancer effects by targeting multiple signaling pathways both in vivo and in vitro. *Int J Cancer* 2012;131:2487-2498.
- 18 Sun W, Modak S: Emerging treatment options for the treatment of neuroblastoma: potential role of perifosine. *Onco Targets Ther* 2012;5:21-29.
- 19 Tong Y, Liu YY, You LS, Qian WB: Perifosine induces protective autophagy and upregulation of ATG5 in human chronic myelogenous leukemia cells in vitro. *Acta Pharmacol Sin* 2012;33:542-550.
- 20 Meng H, Jin Y, Liu H, You L, Yang C, Yang X, Qian W: SNS-032 inhibits mTORC1/mTORC2 activity in acute myeloid leukemia cells and has synergistic activity with perifosine against Akt. *J Hematol Oncol* 2013;6:18.
- 21 Zitzmann K, Vlotides G, Brand S, Lahm H, Spottl G, Goke B, Auernhammer CJ: Perifosine-mediated Akt inhibition in neuroendocrine tumor cells: role of specific Akt isoforms. *Endocr Relat Cancer* 2012;19:423-434.

- 22 Giacomini A, Righi M, Cleris L, Locatelli SL, Mitola S, Daidone MG, Gianni AM, Carlo-Stella C: Induction of death receptor 5 expression in tumor vasculature by perifosine restores the vascular disruption activity of TRAIL-expressing CD34(+) cells. *Angiogenesis* 2013;16:707-722.
- 23 Ji C, Yang YL, Yang Z, Tu Y, Cheng L, Chen B, Xia JP, Sun WL, Su ZL, He L, Bi ZG: Perifosine sensitizes UVB-induced apoptosis in skin cells: new implication of skin cancer prevention? *Cell Signal* 2012;24:1781-1789.
- 24 Zheng K, Lu H, Sheng Z, Li Y, Xu B: Low-concentration of perifosine surprisingly protects cardiomyocytes from oxygen glucose deprivation. *Biochem Biophys Res Commun* 2016;469:753-760.
- 25 Lang E, Lang F: Mechanisms and pathophysiological significance of eryptosis, the suicidal erythrocyte death. *Semin Cell Dev Biol* 2015;39:35-42.
- 26 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.
- 27 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.
- 28 Lau IP, Chen H, Wang J, Ong HC, Leung KC, Ho HP, Kong SK: In vitro effect of CTAB- and PEG-coated gold nanorods on the induction of eryptosis/erythroptosis in human erythrocytes. *Nanotoxicology* 2012;6:847-856.
- 29 Maellaro E, Leoncini S, Moretti D, Del Bello B, Tanganelli I, De Felice C, Ciccoli L: Erythrocyte caspase-3 activation and oxidative imbalance in erythrocytes and in plasma of type 2 diabetic patients. *Acta Diabetol* 2013;50:489-495.
- 30 Bissinger R, Barking S, Alzoubi K, Liu G, Liu G, Lang F: Stimulation of Suicidal Erythrocyte Death by the Antimalarial Drug Mefloquine. *Cell Physiol Biochem* 2015;36:1395-1405.
- 31 Bissinger R, Bouguerra G, Stockinger K, Abbes S, Lang F: Triggering of Suicidal Erythrocyte Death by Topotecan. *Cell Physiol Biochem* 2015;37:1607-1618.
- 32 Bouguerra G, Aljanadi O, Bissinger R, Abbes S, Lang F: Embelin-Induced Phosphatidylserine Translocation in the Erythrocyte Cell Membrane. *Cell Physiol Biochem* 2015;37:1629-1640.
- 33 Bouguerra G, Bissinger R, Abbes S, Lang F: Stimulation of Eryptosis by Narasin. *Cell Physiol Biochem* 2015;37:1807-1816.
- 34 Briglia M, Fazio A, Signoretto E, Faggio C, Lang F: Edelfosine Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2015;37:2221-2230.
- 35 Calabro S, Alzoubi K, Faggio C, Laufer S, Lang F: Triggering of Suicidal Erythrocyte Death Following Boswellic Acid Exposure. *Cell Physiol Biochem* 2015;37:131-142.
- 36 Egler J, Lang F: Licochalcone A Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2015;37:2060-2070.
- 37 Fazio A, Briglia M, Faggio C, Alzoubi K, Lang F: Stimulation of Suicidal Erythrocyte Death by Garcinol. *Cell Physiol Biochem* 2015;37:805-815.
- 38 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.
- 39 Lang E, Zelenak C, Eberhard M, Bissinger R, Rotte A, Ghashghaeinia M, Lupescu A, Lang F, Qadri SM: Impact of Cyclin-Dependent Kinase CDK4 Inhibition on Eryptosis. *Cell Physiol Biochem* 2015;37:1178-1186.
- 40 Lupescu A, Bissinger R, Goebel T, Salker MS, Alzoubi K, Liu G, Chirigiu L, Mack AF, Qadri SM, Lang F: Enhanced suicidal erythrocyte death contributing to anemia in the elderly. *Cell Physiol Biochem* 2015;36:773-783.
- 41 Officioso A, Alzoubi K, Manna C, Lang F: Clofazimine Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2015;37:331-341.
- 42 Peter T, Bissinger R, Enkel S, Alzoubi K, Oswald G, Lang F: Programmed erythrocyte death following in vitro Treosulfan treatment. *Cell Physiol Biochem* 2015;35:1372-1380.
- 43 Stockinger K, Bissinger R, Bouguerra G, Abbes S, Lang F: Enhanced Eryptosis Following Exposure to Carnosic Acid. *Cell Physiol Biochem* 2015;37:1779-1791.
- 44 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.
- 45 Waibel S, Bissinger R, Bouguerra G, Abbes S, Lang F: Saquinavir Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2015;37:1973-1982.
- 46 Zierle J, Bissinger R, Egler J, Lang F: Lapatinib Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2015;37:2275-2287.
- 47 Bissinger R, Waibel S, Bouguerra G, Al Mamun Bhuyan A, Abbes S, Lang F: Enhanced Eryptosis Following Exposure to Lopinavir. *Cell Physiol Biochem* 2015;37:2486-2495.

- 48 Briglia M, Fazio A, Faggio C, Lang F: Triggering of Suicidal Erythrocyte Death by Zosuquidar. *Cell Physiol Biochem* 2015;37:2355-2365.
- 49 Fazio A, Briglia M, Faggio C, Alzoubi K, Lang F: Oxaliplatin Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2015;37:2393-2404.
- 50 Macczak A, Cyrkler M, Bukowska B, Michalowicz J: Eryptosis-inducing activity of bisphenol A and its analogs in human red blood cells (in vitro study). *J Hazard Mater* 2016;307:328-335.
- 51 Officioso A, Manna C, Alzoubi K, Lang F: Bromfenvinphos induced suicidal death of human erythrocytes. *Pestic Biochem Physiol* 2016;126:58-63.
- 52 Qadri SM, Donkor DA, Bhakta V, Eltringham-Smith LJ, Dwivedi DJ, Moore JC, Pepler L, Ivetic N, Nazi I, Fox-Robichaud AE, Liaw PC, Sheffield WP: Phosphatidylserine externalization and procoagulant activation of erythrocytes induced by *Pseudomonas aeruginosa* virulence factor pyocyanin. *J Cell Mol Med* 2016;10.1111/jcmm.12778
- 53 Zierle J, Bissinger R, Bouguerra G, Abbes S, Lang F: Triggering of Suicidal Erythrocyte Death by Regorafenib. *Cell Physiol Biochem* 2016;38:160-172.
- 54 Pagano M, Faggio C: The use of erythrocyte fragility to assess xenobiotic cytotoxicity. *Cell Biochem Funct* 2015;33:351-355.
- 55 Al Mamun Bhuyan A, Signoretto E, Bissinger R, Lang F: Enhanced Eryptosis Following Exposure to Dolutegravir. *Cell Physiol Biochem* 2016;39:639-650.
- 56 Al Mamun Bhuyan A, Signoretto E, Lang F: Triggering of Suicidal Erythrocyte Death by Psammaphin A. *Cell Physiol Biochem* 2016;39:908-918.
- 57 Almasry M, Jemaa M, Mischitelli M, Faggio C, Lang F: Stimulation of Suicidal Erythrocyte Death by Phosphatase Inhibitor Calyculin A. *Cell Physiol Biochem* 2016;40:163-171.
- 58 Bissinger R, Kempe-Teufel DS, Honisch S, Qadri SM, Randrianarisoa E, Haring HU, Henes J, Lang F: Stimulated Suicidal Erythrocyte Death in Arteritis. *Cell Physiol Biochem* 2016;39:1068-1077.
- 59 Egler J, Zierle J, Lang F: Stimulating Effect of Manumycin A on Suicidal Erythrocyte Death. *Cell Physiol Biochem* 2016;38:1147-1156.
- 60 Jemaa M, Mischitelli M, Fezai M, Almasry M, Faggio C, Lang F: Stimulation of Suicidal Erythrocyte Death by the CDC25 Inhibitor NSC-95397. *Cell Physiol Biochem* 2016;40:597-607.
- 61 Lang E, Pozdeev VI, Xu HC, Shinde PV, Behnke K, Hamdam JM, Lehnert E, Scharf RE, Lang F, Haussinger D, Lang KS, Lang PA: Storage of Erythrocytes Induces Suicidal Erythrocyte Death. *Cell Physiol Biochem* 2016;39:668-676.
- 62 Mischitelli M, Jemaa M, Almasry M, Faggio C, Lang F: Stimulation of Erythrocyte Cell Membrane Scrambling by Quinine. *Cell Physiol Biochem* 2016;40:657-667.
- 63 Mischitelli M, Jemaa M, Almasry M, Faggio C, Lang F: Stimulation of Suicidal Erythrocyte Death by Rottlerin. *Cell Physiol Biochem* 2016;40:558-566.
- 64 Mischitelli M, Jemaa M, Almasry M, Faggio C, Lang F: Triggering of Erythrocyte Cell Membrane Scrambling by Emodin. *Cell Physiol Biochem* 2016;40:91-103.
- 65 Mischitelli M, Jemaa M, Almasry M, Faggio C, Lang F: Triggering of Suicidal Erythrocyte Death by Fascaplysin. *Cell Physiol Biochem* 2016;39:1638-1647.
- 66 Mischitelli M, Jemaa M, Almasry M, Faggio C, Lang F: Ca²⁺ Entry, Oxidative Stress, Ceramide and Suicidal Erythrocyte Death Following Diosgenin Treatment. *Cell Physiol Biochem* 2016;39:1626-1637.
- 67 Peter T, Bissinger R, Liu G, Lang F: Anidulafungin-Induced Suicidal Erythrocyte Death. *Cell Physiol Biochem* 2016;38:2272-2284.
- 68 Peter T, Bissinger R, Signoretto E, Mack AF, Lang F: Micafungin-Induced Suicidal Erythrocyte Death. *Cell Physiol Biochem* 2016;39:584-595.
- 69 Pretorius E, du Plooy JN, Bester J: A Comprehensive Review on Eryptosis. *Cell Physiol Biochem* 2016;39:1977-2000.
- 70 Shan F, Yang R, Ji T, Jiao F: Vitamin C Inhibits Aggravated Eryptosis by Hydrogen Peroxide in Glucose-6-Phosphated Dehydrogenase Deficiency. *Cell Physiol Biochem* 2016;39:1453-1462.
- 71 Signoretto E, Castagna M, Al Mamun Bhuyan A, Lang F: Stimulating Effect of Terfenadine on Erythrocyte Cell Membrane Scrambling. *Cell Physiol Biochem* 2016;38:1425-1434.
- 72 Signoretto E, Castagna M, Lang F: Stimulation of Eryptosis, the Suicidal Erythrocyte Death by Piceatannol. *Cell Physiol Biochem* 2016;38:2300-2310.
- 73 Signoretto E, Honisch S, Briglia M, Faggio C, Castagna M, Lang F: Nocodazole Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2016;38:379-392.
- 74 Signoretto E, Laufer SA, Lang F: Stimulating Effect of Sclareol on Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2016;39:554-564.
- 75 Signoretto E, Zierle J, Bissinger R, Castagna M, Bossi E, Lang F: Triggering of Suicidal Erythrocyte Death by Pazopanib. *Cell Physiol Biochem* 2016;38:926-938.

- 76 Wesseling MC, Wagner-Britz L, Huppert H, Hanf B, Hertz L, Nguyen DB, Bernhardt I: Phosphatidylserine Exposure in Human Red Blood Cells Depending on Cell Age. *Cell Physiol Biochem* 2016;38:1376-1390.
- 77 Zierle J, Bissinger R, Lang F: Inhibition by Teriflunomide of Erythrocyte Cell Membrane Scrambling Following Energy Depletion, Oxidative Stress and Ionomycin. *Cell Physiol Biochem* 2016;39:1877-1890.
- 78 Bissinger R, Modicano P, Alzoubi K, Honisch S, Faggio C, Abed M, Lang F: Effect of saponin on erythrocytes. *Int J Hematol* 2014;100:51-59.
- 79 Lang E, Modicano P, Arnold M, Bissinger R, Faggio C, Abed M, Lang F: Effect of thioridazine on erythrocytes. *Toxins (Basel)* 2013;5:1918-1931.
- 80 Al Mamun Bhuyan A, Bissinger R, Cao H, Lang F: Triggering of Suicidal Erythrocyte Death by bexaroten. *Cell Physiol Biochem* 2016;in press.:
- 81 Mischitelli M, Jemaa M, Feza M, Almasry M, Lang F, Faggio C: Stimulation of Erythrocyte Cell Membrane Scrambling by Adarotene. *Cell Physiol Biochem* 2017;41:519-529.
- 82 Bailey HH, Mahoney MR, Ettinger DS, Maples WJ, Fracasso PM, Traynor AM, Erlichman C, Okuno SH: Phase II study of daily oral perifosine in patients with advanced soft tissue sarcoma. *Cancer* 2006;107:2462-2467.
- 83 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.
- 84 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.
- 85 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.
- 86 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.
- 87 Polak-Jonkisz D, Purzyc L: Ca(2+) influx versus efflux during eryptosis in uremic erythrocytes. *Blood Purif* 2012;34:209-210; author reply 210.
- 88 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.
- 89 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.
- 90 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.
- 91 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, Heftner 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.
- 92 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.
- 93 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.
- 94 Andrews DA, Low PS: Role of red blood cells in thrombosis. *Curr Opin Hematol* 1999;6:76-82.
- 95 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.
- 96 Zwaal RF, Comfurius P, Bevers EM: Surface exposure of phosphatidylserine in pathological cells. *Cell Mol Life Sci* 2005;62:971-988.
- 97 Closse C, Dachary-Prigent J, Boisseau MR: Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. *Br J Haematol* 1999;107:300-302.
- 98 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.
- 99 Pandolfi A, Di Pietro N, Siroli 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.
- 100 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.