Naunyn-Schmiedeberg's Archives of Pharmacology (2019) 392:1311–1318
https://doi.org/10.1007/s00210-019-01681-8

ORIGINAL ARTICLE

Triggering of eryptosis, the suicidal erythrocyte death, by phenoxodiol

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Received: 2 April 2019 / Accepted: 19 June 2019 / Published online: 6 July 2019

Abstract

Phenoxodiol is used for the treatment of malignancy. The substance is effective by triggering suicidal tumor cell death or apoptosis. At least in theory, phenoxodiol could similarly stimulate suicidal erythrocyte death or eryptosis. Eryptosis is characterized by cell shrinkage and breakdown of cell membrane asymmetry with phosphatidylserine translocation to the erythrocyte surface. Signaling of eryptosis includes increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), formation of reactive oxygen species (ROS), and increase of ceramide abundance at the cell surface. The present study explored whether phenoxodiol induces eryptosis and whether it modifies Ca^{2+} entry, ROS, and ceramide. Using flow cytometry, phosphatidylserine exposure at the cell surface was quantified from annexin V binding, cell volume from forward scatter, $[Ca^{2+}]_i$ from Fluo3 fluorescence, ROS from DCFDA-dependent fluorescence, and ceramide abundance utilizing specific antibodies. A 48-h exposure of human erythrocytes to phenoxodiol (100 μg/ml [416 μM]) significantly increased the percentage of annexin V binding cells, significantly decreased average forward scatter and Fluo3 fluorescence and significantly increased ceramide abundance, but did not significantly modify DCFDA fluorescence. The effect of phenoxodiol on annexin V binding tended to decrease following removal of extracellular Ca^{2+}, an effect, however, not reaching statistical significance. In conclusion, phenoxodiol triggers eryptosis, an effect paralleled by increase of ceramide abundance.

Keywords Phosphatidylserine · Eryptosis · Staurosporine · Ceramide · Oxidative stress · Calcium · Red blood cells

Introduction

Phenoxodiol, an isoflavonoid (Fig. 1), is utilized in the treatment of malignancy (Aguero et al. 2005; Alvero et al. 2006; Choueiri et al. 2006b; Mor et al. 2006; Saif et al. 2009; Silasi et al. 2009; de Souza et al. 2010). The substance is at least in part effective by triggering suicidal death and sensitization against cytoxic treatment of tumor cells (Kamsteeg et al. 2003; Sapi et al. 2004; Straszewski-Chavez et al. 2004; Alvero et al. 2006, 2008; Choueiri et al. 2006a, b; Gamble et al. 2006; Mor et al. 2006, 2008; Yu et al. 2006; Herst et al. 2007, 2009; Kluger et al. 2007; Morre et al. 2007; Yagiz et al. 2007; De Luca et al. 2008, 2010; Aguero et al. 2010; Wu et al. 2011; Mahoney et al. 2012; Yao et al. 2012; Li et al. 2014; Isono et al. 2018; Miyamoto et al. 2018). Studies on structure-activity relationships (Silasi et al. 2009; Chen et al. 2015) revealed that the anticancer activity of phenoxodiol was superior to the parent molecule genistein (Silasi et al. 2009). Studies utilizing tumor xenograft mouse models demonstrated in vivo activity of phenoxodiol (Alvero et al. 2006, 2007; McPherson et al. 2009; Aguero et al. 2010; Yao et al. 2012; Li et al. 2014).

At least in theory, phenoxodiol could similarly trigger eryptosis, the suicidal death of erythrocytes characterized by breakdown of cell membrane asymmetry with phosphatidylserine translocation to the cell surface (Mischitelli et al. 2016a; Bissinger et al. 2019) and cell shrinkage (Lang et al. 2003). Signaling regulating eryptosis includes increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) (Mischitelli et al. 2016b; Bissinger et al. 2019), ceramide appearance at the cell surface (Abed et al. 2012), and oxidative stress (Bissinger et al. 2019). Triggers of eryptosis include diverse cytotoxic

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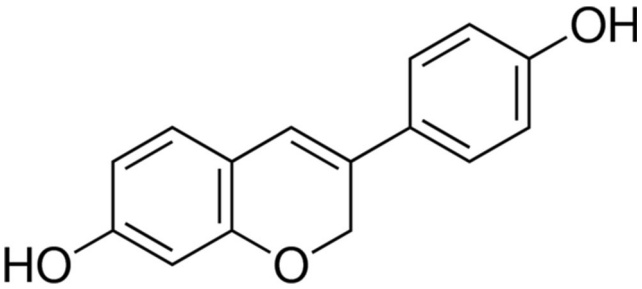

Fig. 1 Structure of phenoxodiol (Sigma)

drugs (Lang et al. 2013, 2017; Pretorius et al. 2016; Briglia et al. 2017; Bissinger et al. 2019).

The present study explored whether phenoxodiol is capable of stimulating eryptosis. Erythrocytes isolated from healthy volunteers were treated with phenoxodiol and phosphatidylserine surface abundance, cell volume, $[Ca^{2+}]_i$, ROS formation, and ceramide abundance were determined by flow cytometry.

Materials and methods

Erythrocytes, solutions, and chemicals

Erythrocytes were isolated from fresh Li-heparin-anticoagulated blood samples drawn from healthy volunteers in the blood bank of the University Clinic 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, platelets and leukocytes-containing supernatant discarded, and erythrocytes incubated 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, and 1 $CaCl_2$, at 37 °C for 48 h. In nominally Ca^{2+}-free solutions, 1 mM $CaCl_2$ was replaced by 1 mM EGTA. Where indicated, erythrocytes were exposed for 48 h to phenoxodiol (Sigma, Schnelldorf, Germany) at concentrations ranging from 25 μg/ml (104 μM) to 500 μg/ml (2081 μM).

Annexin V binding and forward scatter

Erythrocytes 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 protected against light. Annexin V abundance at the erythrocyte surface was quantified in a FACSCalibur (BD, Heidelberg, Germany) with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Parallel forward scatter (FSC) was determined as measure of cell volume.

Intracellular Ca^{2+}

After incubation and washing in Ringer solution, erythrocytes were loaded with Fluo-3/AM (Biotium, Hayward, USA) at 37 °C for 30 min in Ringer solution containing 5 mM $CaCl_2$ and 5 μM Fluo-3/AM. Fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACSCalibur. The geomean of the Ca^{2+}-dependent fluorescence was calculated.

Reactive oxygen species

Reactive oxygen species (ROS) was determined utilizing 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, washing in Ringer solution, and staining with 10 μM DCFDA (Sigma, Schnelldorf, Germany) in Ringer solution, erythrocytes were incubated at 37 °C for 30 min in the dark and washed two times in Ringer solution. ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACSCalibur (BD). The geomean of the DCFDA-dependent fluorescence was calculated.

Ceramide abundance

Ceramide abundance at the erythrocyte surface was quantified with a monoclonal antibody-based assay. Erythrocytes 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 1:10, washed twice with PBS-BSA, and stained for 30 min with polyclonal fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG- and IgM-specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. FITC abundance was quantified by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The geomean of the ceramide-dependent fluorescence was calculated.

Statistics

Measured values are expressed as arithmetic means ± SD. Statistical analysis was made using ANOVA with Tukey's test as post-test and *t* test as appropriate (n = number of different erythrocyte specimens studied).

Results

The effect of phenoxodiol on eryptosis was tested by determination of cell membrane scrambling and cell shrinkage.

Erythrocytes were incubated for 48 h in Ringer solution without or with phenoxodiol (25–100 μg/ml [104–416 μM] or 100–500 μg/ml [416 μM–2080 μM]) and cell membrane scrambling quantified from phosphatidylserine abundance at the erythrocyte surface identified by determination of annexin V binding utilizing flow cytometry. As illustrated in Fig. 2, a 48-h exposure to phenoxodiol increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 50 μg/ml (208 μM) phenoxodiol.

Erythrocyte shrinkage was quantified by measuring erythrocyte forward scatter utilizing flow cytometry. As a result, exposure to phenoxodiol (25–100 μg/ml [104–416 μM] or 100–500 μg/ml [416 μM–2080 μM]) decreased the average erythrocyte forward scatter, an effect reaching statistical significance at 50 μg/ml (208 μM) phenoxodiol (Fig. 3).

A next series of experiments addressed cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). Fluo3 fluorescence was employed in order to quantify $[Ca^{2+}]_i$. The erythrocytes were analyzed after a 48-h incubation in Ringer solution without or with phenoxodiol (25–100 μg/ml [104–416 μM]). As illustrated in Fig. 4, a 48-h exposure to phenoxodiol (25–100 μg/ml [104–416 μM]) decreased the Fluo3 fluorescence, an effect reaching statistical significance at each 25, 50, and 100 μg/ml (104, 208, 416 μM) phenoxodiol.

Further experiments explored whether phenoxodiol-induced cell membrane scrambling required entry of extracellular Ca^{2+}. To this end, erythrocytes were incubated for 48 h in the absence or presence of 100 μg/ml (416 μM) phenoxodiol in the presence or nominal absence of extracellular Ca^{2+}. As shown in Fig. 5, removal of extracellular Ca^{2+} did not significantly modify the effect of phenoxodiol on annexin V binding. Both in the presence and absence of extracellular Ca^{2+} did phenoxodiol significantly increase the percentage of annexin V binding erythrocytes. Accordingly, the phenoxodiol-induced cell membrane scrambling did not require entry of extracellular Ca^{2+}.

ROS was determined utilizing DCFDA. As illustrated in Fig. 6, a 48-h exposure to phenoxodiol (100 μg/ml [416 μM]) did not significantly modify the DCFDA fluorescence of erythrocytes. Accordingly, phenoxodiol did not appreciably induce oxidative stress.

Ceramide abundance at the erythrocyte surface was determined utilizing specific antibodies. As shown in Fig. 7, a 48-h exposure to phenoxodiol (100 μg/ml [416 μM]) significantly increased the ceramide abundance at the erythrocyte surface.

Discussion

The present study discloses a novel effect of phenoxodiol, i.e., the stimulation of eryptosis, the suicidal erythrocyte death, which is characterized by erythrocyte shrinkage and erythrocyte membrane scrambling with phosphatidylserine translocation to the erythrocyte surface (Bissinger et al. 2019). The phenoxodiol concentrations required for significant stimulation of eryptosis are in

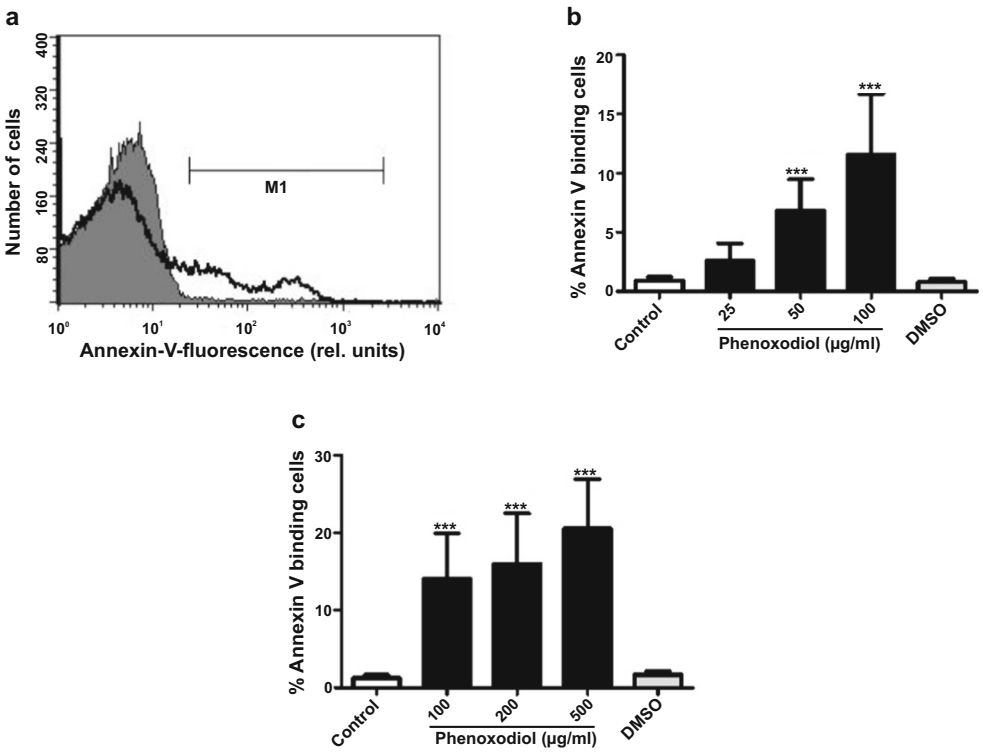

Fig. 2 Effect of phenoxodiol on phosphatidylserine exposure. **a** Histogram of annexin V binding of erythrocytes following exposure for 48 h to Ringer solution without (gray area) and with (black line) presence of 100 μg/ml (416 μM) phenoxodiol. **b** Arithmetic means ± SD ($n = 20$) of erythrocyte annexin V binding following incubation for 48 h to Ringer solution without (white bar) or with (black bars) phenoxodiol (25–100 μg/ml [104–416 μM]) or the solvent alone (DMSO, gray bar). **c** Arithmetic means ± SD ($n = 15$) of erythrocyte annexin V binding following incubation for 48 h to Ringer solution without (white bar) or with (black bars) phenoxodiol (100–500 μg/ml [416–2080 μM]) or the solvent alone (DMSO, gray bar). ***$p < 0.001$ indicates significant difference from the absence of phenoxodiol (ANOVA)

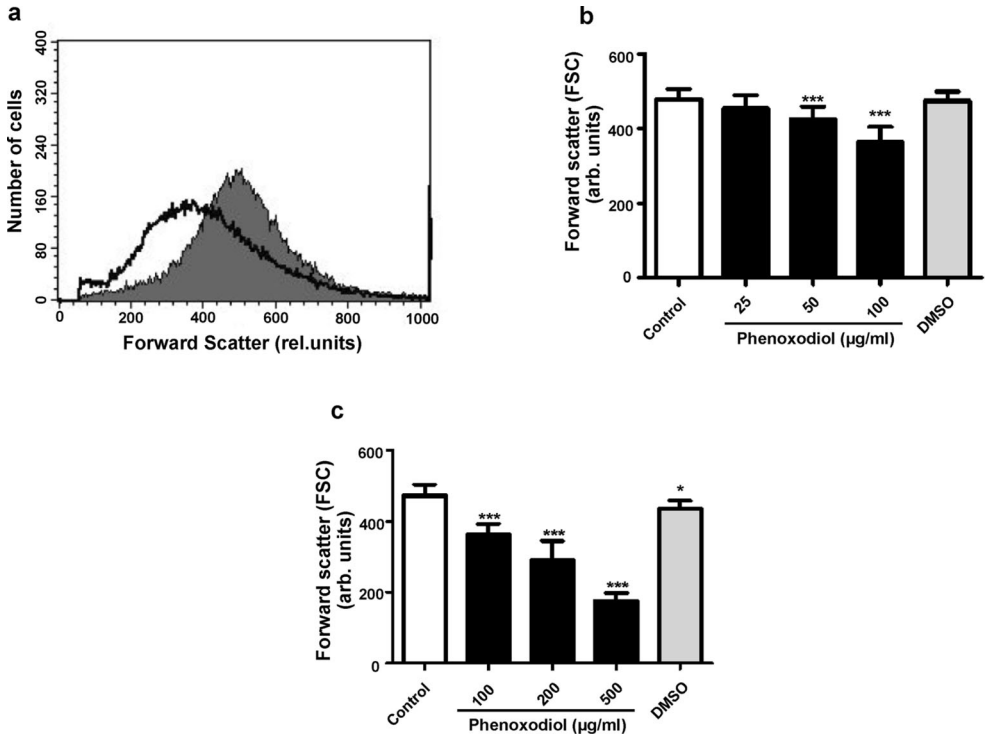

Fig. 3 Effect of phenoxodiol on erythrocyte forward scatter. **a** Histograms of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (gray area) and with (black line) presence of 100 μg/ml (416 μM) phenoxodiol. **b** Arithmetic means ± SD ($n = 27$) of the erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) phenoxodiol (25–100 μg/ml [104–416 μM]) or the solvent alone (DMSO, gray bar). **c** Arithmetic means ± SD ($n = 15$) of the erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) phenoxodiol (100–500 μg/ml [416–2080 μM]) or the solvent alone (DMSO, gray bar). ***$p < 0.001$ indicates significant difference from the absence of phenoxodiol (ANOVA)

the range of concentrations reported in the plasma of patients under phenoxodiol treatment (Choueiri et al. 2006a). A linear correlation was observed between dosage and plasma concentrations reaching 60 μg/ml (250 μM) plasma concentration at a dosage of 27 mg/kg/24 h (Choueiri et al. 2006a).

The present study further sheds some light on the signaling involved in the stimulation of eryptosis by phenoxodiol. The effect of phenoxodiol on cell membrane scrambling was paralleled by an increase of ceramide abundance at the erythrocyte surface. Ceramide is a well-known stimulator of cell membrane scrambling. Ceramide is

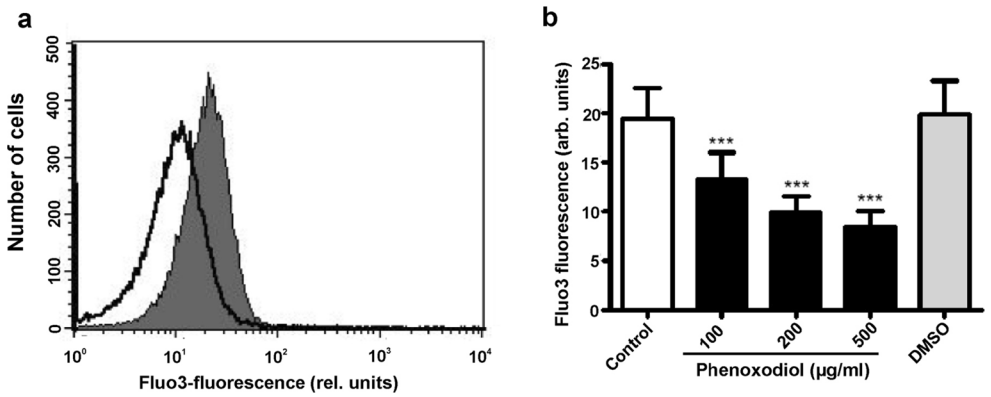

Fig. 4 Effect of phenoxodiol on cytosolic Ca^{2+} activity. **a** Histogram of Fluo3 fluorescence of erythrocytes following exposure for 48 h to Ringer solution without (gray area) and with (black line) presence of 100 μg/ml (416 μM) phenoxodiol. **b** Arithmetic means ± SD ($n = 27$) of erythrocyte Fluo3 fluorescence following incubation for 48 h to Ringer solution without (white bar) or with (black bars) phenoxodiol (25–100 μg/ml [104–416 μM]) or the solvent alone (DMSO, gray bar). ***$p < 0.001$ indicates significant difference from the absence of phenoxodiol (ANOVA)

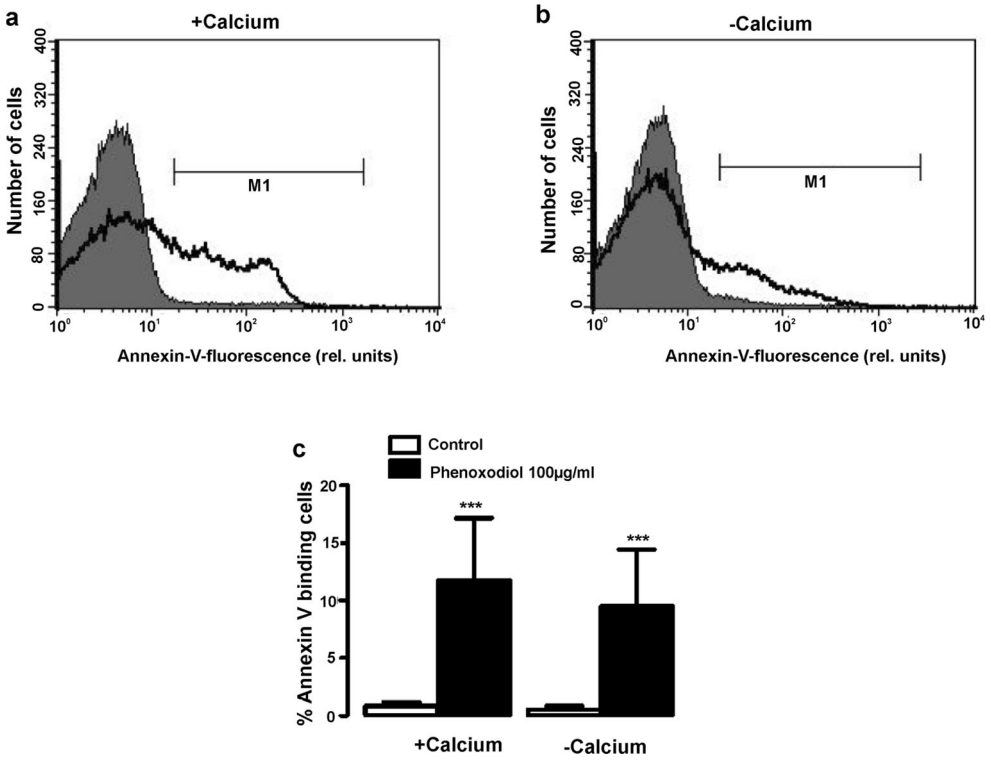

Fig. 5 Ca^{2+} sensitivity of phenoxodiol-induced phosphatidylserine exposure. **a**, **b** Histograms of annexin V binding of erythrocytes following exposure for 48 h to Ringer solution without (gray areas) and with (black lines) phenoxodiol (100 μg/ml [416 μM]) in the presence (**a**) and absence (**b**) of extracellular Ca^{2+}. **c** Means ± SD ($n = 16$) of annexin V binding of erythrocytes after a 48-h treatment with Ringer solution without (white bars) or with (black bars) phenoxodiol (100 μg/ml [416 μM]) 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 phenoxodiol (ANOVA)

partially effective by sensitizing erythrocytes for the scrambling effect of Ca^{2+} (Bissinger et al. 2019). Phenoxodiol decreases cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) and the effect of phenoxodiol on cell membrane scrambling was not significantly modified by removal of extracellular Ca^{2+}. The phenoxodiol-induced eryptosis apparently does not depend on Ca^{2+} entry. Along those lines, phenoxodiol triggered cell membrane scrambling even in the nominal absence of extracellular Ca^{2+}. Phenoxodiol did not significantly modify the abundance of reactive oxygen species, another stimulator of eryptosis (Bissinger et al. 2019). The present observations, however, do not rule out the involvement of further signaling pathways. The present study does not define the direct molecular target of phenoxodiol. Ceramide is produced by acid sphingomyelinase, which is upregulated by platelet activating factor (PAF) (Lang et al. 2015). The cation channel is activated by prostaglandin E2, which is generated by cyclo-oxygenase (Lang et al. 2005). Possibly, phenoxodiol influences ceramide abundance and channel activity by directly or indirectly influencing PAF formation

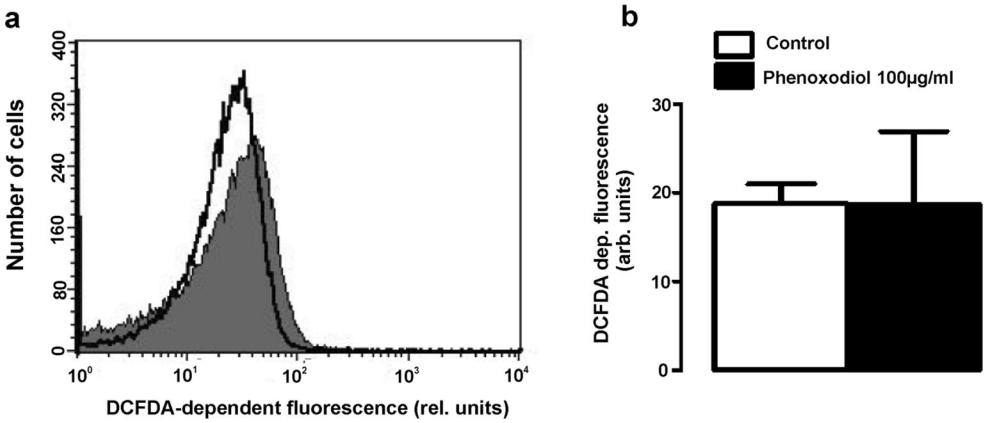

Fig. 6 Effect of phenoxodiol on reactive oxygen species. **a** Histogram of DCFDA fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (gray area) and with (black line) presence of 100 μg/ml (416 μM) phenoxodiol. **b** Arithmetic means ± SD ($n = 22$) of DCFDA fluorescence in erythrocytes following incubation for 48 h to Ringer solution without (white bar) or with (black bar) phenoxodiol (100 μg/ml [416 μM]). ***$p < 0.001$ indicates significant difference from the absence of phenoxodiol (ANOVA)

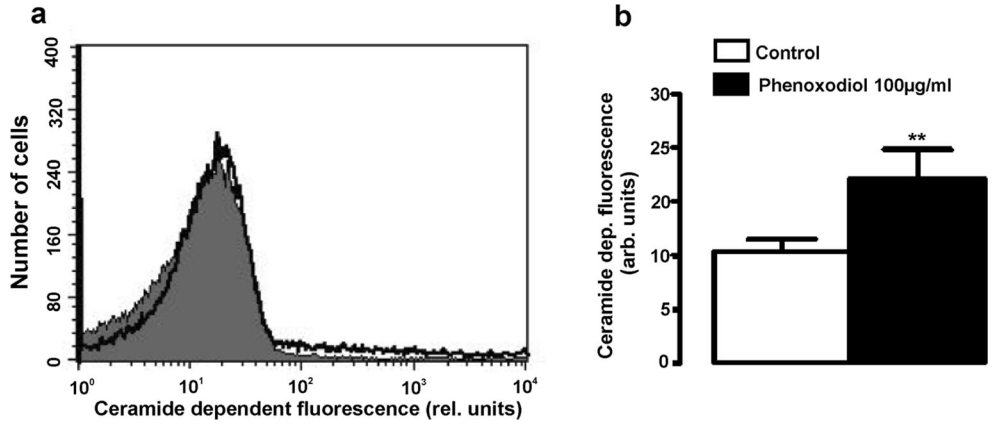

Fig. 7 Effect of phenoxodiol on ceramide abundance. **a** Histogram of ceramide abundance in erythrocytes following exposure for 48 h to Ringer solution without (gray area) and with (black line) presence of 100 μg/ml (416 μM) phenoxodiol. **b** Arithmetic means ± SD ($n = 13$) of ceramide abundance in erythrocytes following incubation for 48 h to Ringer solution without (white bar) or with (black bar) phenoxodiol (100 μg/ml [416 μM]). $^{**}p < 0.01$ indicates significant difference from the absence of phenoxodiol (unpaired t test)

and cyclo-oxygenase activity. Several signaling pathways further have been shown to be sensitive to phenoxodiol (Table 1).

The limitations of the present study include the lack of in vivo data and the uncertainty of the direct molecular target. Future studies may explore the influence of phenoxodiol on

Table 1 Reported effects of phenoxodiol

Effect	Concentration	Cell type	References
• Caspase-2-dependent activation of Bid engaging the mitochondrial pathway leading to the release of cytochrome c, Smac/DIABLO, and Omi/HtrA2 • Increased activity of caspase-3, caspase-8, caspase-9 • Proteasomal degradation of the anti-apoptotic protein XIAP • Decreased FLIP-expression via decreased Akt expression • Increase of sensitivity to Fas-mediated apoptosis due to inactivation of FLIP and XIAP	1–10 μg/ml (4–42 μM)	Ovarian carcinoma	Kamsteeg et al. (2003), Alvero et al. (2006)
Cell cycle arrest in the G1/S phase: • Via p21 upregulation • Partially via increased expression of cMyc • Partially via decreased expression of cyclin-D1 • Partially via decreased Ki-67 expression	≥ 2 μg/ml (≥ 10 μM)	Prostate cancer cells	Mahoney et al. (2014)
• G_1 arrest via p53-independent induction of p21 leading to specific loss in cyclin-dependent kinase 2 activity	≥ 5 μg/ml (21 μM)	Head and neck cancer cells	Aguero et al. (2005)
• Reduced XIAP-levels, increased caspase-2 activation, increased activity of caspase-3, caspase-8, and caspase-9 • Induction of p53-dependent BH3 proteins (PUMA, NOXA) • Induction of the p53-independent Bim protein resulting in BAX activation	10 μg/ml (42 μM) 2 μg/ml (8 μM)	Melanoma cells	Yu et al. (2006), Kluger et al. (2007)
Inhibition of proliferation, migration, and capillary tube formation of endothelial cells via: • Inhibited expression of adhesion molecuses such as E-selectin and VCAM-1 • Downregulation of enzymes such as MMP-2 required for matrix breakdown • Inhibition of lipid kinase SK, implicated in endothelial activation and angiogenesis	10 μg/ml (42 μM)	Human umbilical vein endothelial cells	Gamble et al. (2006)
• Inhibition of plasma membrane electron transport (PMET) • Increased cell surface NADH-oxidase activity • Inhibition of cell proliferation	24 μg/ml (≤ 100 μM)	Human leukemic HL60 cells, activated splenic T cells	Herst et al. (2007)
• Potent chemosensitizer of most standard chemotherapeutics: platinum, gemcitabine, taxanes, doxorubicin • Reversing chemoresistance of tumor cells	5–20 μg/ml (21–83 μM)	Ovarian cancer cells, melanoma cells, osteosarcoma cells, gall bladder cancer	Alvero et al. (2006), Kluger et al. (2007), Yao et al. (2012), Li et al. (2014)

blood count and packed cell volume in vivo, and the influence of the drug on PAF (Lang et al. 2015) and/or cyclooxygenase activity (Lang et al. 2005). Moreover, future studies may address the possibility that phenoxodiol may favorably influence the clinical course of malaria by sensitizing erythrocytes for the eryptotic effect of *Plasmodium* infection and thus eryptosis and clearance of infected erythrocytes (Foller et al. 2009).

In conclusion, phenoxodiol triggers erythrocyte cell membrane scrambling, an effect paralleled by increase of ceramide abundance.

Acknowledgments The authors acknowledge the meticulous preparation of the manuscript by Lejla Subasic.

Authors' contribution BN, CF, and FL conceived and designed research. MF and AMB conducted experiments and analyzed data. FL wrote the manuscript. All authors read and approved the manuscript.

Funding The study was supported by the Deutsche Forschungsgemeinschaft, DFG grant "G_i proteins and platelets" (NU 53/13-1) and Deutscher Akademischer Austaauschdienst (DAAD).

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