Enhanced Eryptosis Following Auranofin Exposure

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Key Words
Phosphatidylserine • Auranofin • Calcium • Cell volume • ROS • Oxidative stress • Eryptosis

Abstract
Background/Aims: The antiinflammatory, antimicrobial and anticancer drug auranofin has previously been shown to trigger apoptosis, the suicidal death of nucleated cells. Side effects of the drug include anaemia. At least in theory the anaemia could result from stimulation of suicidal death of erythrocytes or eryptosis, which involves cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Methods: Stimulators of eryptosis include oxidative stress and increase of cytosolic Ca$^{2+}$-activity ([Ca$^{2+}$]). In the present study, phosphatidylserine exposure at the cell surface was estimated from annexin V binding, cell volume from forward scatter, hemolysis from hemoglobin release, reactive oxygen species (ROS) from 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence, and [Ca$^{2+}$]$_i$ from Fluo3-fluorescence. Results: A 24 hours exposure of human erythrocytes to auranofin (≥5 µg/ml) significantly increased the percentage of annexin-V-binding cells (from 2.2 ± 0.5 to 17.4 ± 1.5%), significantly decreased forward scatter and significantly enhanced ROS. At higher concentrations (10 µg/ml) auranofin triggered slight hemolysis (from 2.1 ± 0.2 to 3.2 ± 0.3%). Conclusions: Auranofin stimulates cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect at least partially due to induction of oxidative stress.

Introduction

Auranofin has initially been used as an anti-inflammatory drug in the treatment of rheumatoid arthritis and psoriasis [1-5], but is more recently used for the treatment of malignancy [1, 2, 6, 7] as well as parasitic [2, 8, 9], bacterial [2, 10], and viral infections [2, 11, 12].

The anticancer effect of auranofin results at least in part from its ability to trigger apoptosis of tumor cells [13-16]. The proapoptotic effect of auranofin has been attributed to increase of cytosolic Ca$^{2+}$ activity [17], thioredoxin (Trx)-2 reductase inhibition, GSH decline

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and oxidative stress [18-25], p38 protein kinase activation [26], FOXO3 activation [27], as well as annexin V expression and translocation [28]. Moreover, auranofin has been shown to inhibit PGE$_2$ formation [5].

Side effects of auranofin treatment include anemia [29-32]. At least in theory the anemia could result from stimulation of apoptosis-like suicidal cell death or eryptosis, which is characterized by cell shrinkage [33] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [34]. As gold has previously been shown to trigger eryptosis [35-37], an eryptotic effect of auranofin appeared likely, even though auranofin does not necessarily share all effects with other gold preparations.

Eryptosis may be triggered by oxidative stress and/or weakening of antioxidant defence [34], Ca$^{2+}$ entry with increase of cytosolic Ca$^{2+}$ activity ([Ca$^{2+}$]$_i$), ceramide [38], energy depletion [34], activated caspases [34, 36, 39], activation of casein kinase 1α, Janus-activated kinase JAK3, protein kinase C, p38 kinase and PAK2 kinase [34], as well as inhibition or genetic defects of AMP activated kinase AMPK, cGMP-dependent protein kinase, and sorafenib/sunitinib sensitive kinases [34]. Eryptosis may be stimulated by diverse xenobiotics [34,40-68].

The present study tested, whether auranofin is able to stimulate eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to auranofin and phosphatidylserine surface abundance and cell volume determined by flow cytometry. As a matter of fact, auranofin indeed triggered eryptosis. Additional experiments addressed the mechanisms involved, such as reactive oxygen species and [Ca$^{2+}$]$_i$.

**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), 5 glucose, 1 CaCl$_2$; pH 7.4 at 37°C for 24 h. Where indicated, erythrocytes were exposed to auranofin (Sigma Aldrich, Hamburg, Germany) at the indicated concentrations.

**Annexin-V-binding and forward scatter**

After incubation under the respective experimental condition, 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. In the following, the forward scatter (FSC) of the cells was determined, and annexin-V-FITC fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany). In order to avoid counting cellular debris, a cutoff value of 52 was placed for forward scatter. In order to define annexin-V-binding cells, a marker (M1) was placed at the right border of the unstained population (non apoptotic cells).

**Measurement of 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 twice in Ringer solution containing 5 mM CaCl₂. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer. Then, Ca²⁺-dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

**Reactive oxidant species (ROS)**

Oxidative stress was determined utilizing 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA). The substance has been used to trace the oxidant status of erythrocytes [69]. After incubation, a 100 µl suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA (Sigma, Schnelldorf, Germany) in Ringer solution containing DCFDA at a final concentration of 10 µM. Erythrocytes were incubated at 37°C for 30 min in the dark and then washed three times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 µl 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).

**Statistics**

Data are expressed as arithmetic means ± SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and t test as appropriate. n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

**Results**

The present study addressed the possibility that auranofin triggers eryptosis, the suicidal erythrocyte death, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface.

Phosphatidylserine at the erythrocyte surface was identified by annexin-V-binding, which was quantified by flow cytometry. Prior to measurement of annexin-V-binding the erythrocytes were incubated for 24 hours in Ringer solution without or with auranofin (0.5 - 10 µg/ml). As shown in Fig. 1, a 24 hours exposure to auranofin increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 5 µg/ml
auranofin concentration. Auranofin treatment thus triggers erythrocyte cell membrane scrambling with translocation of phosphatidylserine to the cell surface.

Additional experiments addressed the putative effect of auranofin on hemolysis, a cell death distinct from eryptosis. To this end, the hemoglobin concentration in the supernatant was determined by photometry. As illustrated in Fig. 1, a 24 hours incubation with auranofin (0.5 - 10 µg/ml) had only little stimulating effect on hemolysis, reaching statistical significance at 10 µg/ml auranofin concentration. The percentage hemolysed erythrocytes remained, however, one order of magnitude smaller than the percentage erythrocytes binding annexin-V.

Forward scatter in flow cytometry was employed to estimate erythrocyte cell volume, which was determined following a 24 hours incubation in Ringer solution without or with auranofin (0.5 - 10 µg/ml). As illustrated in Fig. 2, incubation in Ringer solution with auranofin decreased erythrocyte forward scatter, an effect reaching statistical significance at 5 µg/ml auranofin concentration.

Fig. 2. Effect of auranofin 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 auranofin. (B) Arithmetic means ± SEM (n = 12) of the normalized erythrocyte forward scatter (FSC) following incubation for 24 hours to Ringer solution without (white bar) or with (black bars) auranofin (0.5 - 10 µg/ml). *** (p<0.001) indicate significant difference from the absence of auranofin (ANOVA).

Fig. 3. Effect of auranofin on erythrocyte Ca²⁺ 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 auranofin (10 µg/ml). (B) Arithmetic means ± SEM (n = 12) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 24 hours to Ringer solution without (white bar) or with (black bars) auranofin (0.5 - 10 µg/ml).
Alzoubi et al.: Auranofin-Induced Eryptosis

Cellular Physiology
and Biochemistry

A further series of experiments explored, whether auranofin modifies cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)\]). To this end Fluo3 fluorescence was employed to quantify [Ca\(^{2+}\)], As shown in Fig. 3, a 24 hours exposure to auranofin (0.5 – 10 µg/ml) did not significantly modify Fluo3 fluorescence and was thus without appreciable effect on cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)])

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was quantified utilizing 2′, 7′-dichlorodihydrofluorescein diacetate (DCFDA). As illustrated in Fig. 4, a 24 hours exposure to auranofin (10 µg/ml) significantly increased the DCFDA fluorescence, an observation pointing to induction of oxidative stress.

A further series of experiments explored whether auranofin-induced cell membrane scrambling was dependent on oxidative stress. To this end, erythrocytes were exposed for 24 hours to 10 µg/ml auranofin in the absence or presence of the antioxidant N-acetyl-cysteine (2 mM). As a result, the increase of percentage annexin-V-binding erythrocytes was similar in the absence of skepinone (from 0.8 ± 0.1% to 29.3 ± 2.7%, n = 12) as in the presence of skepinone (from 1.1 ± 0.1% to 26.4 ± 3.0%, n = 12).

A further series of experiments explored whether auranofin-induced cell membrane scrambling was dependent on activation of p38 kinase. To this end, erythrocytes were exposed for 24 hours to 10 µg/ml auranofin in the absence or presence of the p38 kinase inhibitor skepinone at a concentration (2 µM) known to inhibit p38 kinase [70]. As a result, the increase of percentage annexin-V-binding erythrocytes was similar in the absence of skepinone (from 0.8 ± 0.1% to 29.3 ± 2.7%, n = 12) as in the presence of skepinone (from 1.1 ± 0.1% to 26.4 ± 3.0%, n = 12).

Discussion

The present study uncovers a novel effect of auranofin, i.e. the stimulation of eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The auranofin concentration (5 µg/ml) required for stimulation of erythrocyte cell membrane scrambling was within the range of concentrations required for stimulation of tumor cell apoptosis [24,
Auranofin induced eryptosis was apparently not due to increase of cytosolic Ca$^{2+}$ activity ([Ca$^{2+}$]). An increase of [Ca$^{2+}$] is known to trigger cell membrane scrambling [34] and to activate Ca$^{2+}$ sensitive K$^+$ channels leading to cell shrinkage due to K$^+$ exit, cell membrane hyperpolarization, CI$^-$ exit and thus cellular loss of KCl with osmotically obliged water [33]. According to Fluo3 fluorescence, auranofin did not appreciably increase [Ca$^{2+}$] $i$. The observations do not exclude, however, minor alterations of [Ca$^{2+}$]. In nucleated cells auranofin has been shown to increase cytosolic Ca$^{2+}$ activity [17].

The stimulation of cell membrane scrambling is at least in part the result of oxidative stress, a well known trigger of suicidal erythrocyte death [34]. In nucleated cells auranofin has been shown to lower thioredoxin (Trx)-2 reductase activity, to decrease antioxidant GSH and to induce oxidative stress [18-25]. In erythrocytes the effect of auranofin was virtually abolished by the antioxidant N-acetyl-cysteine, an observation highlighting the significance of oxidative stress in the triggering of eryptosis by auranofin.

In nucleated cells auranofin has been shown to be effective in part by p38 protein kinase activation [26]. The auranofin induced eryptosis was, however, not significantly modified by p38 kinase inhibitor skepinone.

Eryptosis accomplishes the disposal of defective erythrocytes prior to hemolysis [34] thus preventing release of hemoglobin, which is otherwise filtered in the kidney, precipitates in the acidic lumen of renal tubules and thus occludes the respective nephrons [73]. Eryptosis further counteracts parasitemia and thus favourably influences the clinical course of malaria [74]. Oxidative stress imposed on the infected erythrocyte by the malaria pathogen Plasmodium activates host cell ion channels including Ca$^{2+}$-permeable erythrocyte cation channels [34, 75]. Ca$^{2+}$ entry through those channels triggers eryptosis with subsequent clearance of infected erythrocytes from circulating blood [74]. Sickle-cell trait, beta-thalassemia-trait, homozygous Hb-C and homozygous G6PD-deficiency protect against a severe course of malaria at least partially by increasing the erythrocyte susceptibility to triggers of eryptosis [34, 76-78]. Stimulation of eryptosis further counteracts parasitemia in iron deficiency [79], and treatment with lead [79], chlorpromazine [80] or NO synthase inhibitors [80]. In theory, auranofin may favourably influence the clinical course of malaria by augmentation of oxidative stress and eryptosis in plasmodium infected erythrocytes.

Excessive eryptosis with subsequent clearance of phosphatidylserine exposing erythrocytes may, however, lead to anemia, if the removal of eryptotic erythrocytes cannot be matched by an equivalent increase of erythropoiesis [34]. Phosphatidylserine exposing erythrocytes may further adhere to the vascular wall [81], trigger blood clotting and elicit thrombosis [82-84]. Eryptotic erythrocytes may thus impair microcirculation [38, 82, 85-88]. The effect of auranofin may be augmented in clinical conditions associated with enhanced susceptibility to eryptosis, such as dehydration [56], hyperphosphatemia [67], chronic kidney disease (CKD) [45, 89-91], hemolytic-uremic syndrome [92], diabetes [93], hepatic failure [94], malignancy [95], sepsis [96], Sickle-cell disease [97], beta-thalassemia [97], Hb-C deficiency [97], G6PD-deficiency [97] and Wilsons disease [98].

**Conclusion**

In conclusion, auranofin triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect paralleled by and at least partially caused by oxidative stress.

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Disclosure Statement

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

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