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Original Paper

Stimulation of Erythrocyte Cell Membrane Scrambling by Mitotane

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Key Words
Phosphatidylserine • Ionomycin • Calcium • Cell volume • Eryptosis

Abstract
Background: Mitotane (1,1-dichloro-2-[o-chlorophenyl]-2-[p-chlorophenyl]ethane), a cytostatic drug used for the treatment of adrenocortical carcinomas, is effective by triggering tumor cell apoptosis. In analogy to apoptosis of nucleated cells, eryptosis is the suicidal death of erythrocytes, which is typically paralleled by cell shrinkage and breakdown of cell membrane phosphatidylserine asymmetry with subsequent phosphatidylserine exposure at the erythrocyte surface. Eryptosis may be triggered by increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). The present study tested, whether treatment of human erythrocytes with mitotane is followed by eryptosis. ***Methods:*** $[Ca^{2+}]_i$ was estimated from Fluo3 fluorescence, cell volume from forward scatter, phosphatidylserine exposure from annexin V binding, and hemolysis from hemoglobin release. ***Results:*** Exposure to mitotane (≥ 5 µg/ml ≈ 16 µM) significantly increased $[Ca^{2+}]_i$, increased annexin V binding and triggered hemolysis, but did not significantly modify forward scatter. The effect on annexin V binding was significantly blunted in the absence of extracellular Ca^{2+}. Within 30 min Ca^{2+} ionophore ionomycin (1 µM) decreased forward scatter, an effect virtually abolished in the presence of mitotane (15 µg/ml). ***Conclusions:*** Mitotane increases $[Ca^{2+}]_i$ with subsequent phosphatidylserine translocation. By the same token mitotane inhibits Ca^{2+} induced cell shrinkage.

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Introduction

Mitotane (1,1-dichloro-2-[o-chlorophenyl]-2-[p-chlorophenyl]ethane) is the drug of choice for non-resectable and metastatic adrenocortical carcinoma, a rare malignancy with a 5-year survival of less than 50% [1-3]. The substance is effective by triggering apoptosis of tumor cells [4-6], an effect attributed to caspase activation [4]. Potential side effects of mitotane include anemia [7, 8].

Similar to apoptosis of nucleated cells, erythrocytes may undergo eryptosis, the suicidal death of erythrocytes characterized by erythrocyte shrinkage and breakdown of phosphatidylserine (PS) asymmetry of the erythrocyte cell membrane [9]. Eryptosis is stimulated by increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) resulting from Ca^{2+} entry through Ca^{2+} permeable cation channels [9]. Increased $[Ca^{2+}]_i$ activates Ca^{2+} sensitive K^+ channels [10] resulting in K^+ efflux, hyperpolarization, Cl^- efflux and thus cell shrinkage due to cellular efflux of KCl with osmotically obliged water [9]. Increased $[Ca^{2+}]_i$ further triggers phospholipid scrambling of the cell membrane with translocation of phosphatidylserine to the erythrocyte surface [11]. The Ca^{2+} sensitivity of eryptosis is augmented by ceramide [9]. Further stimulators of eryptosis include energy depletion [9] and caspase activation [9, 12, 13]. Signaling governing eryptosis further includes AMP activated kinase AMPK [9], cGMP dependent protein kinase [9], Janus activated kinase JAK3 [14], casein kinase [15, 16], p38 kinase [17], PAK2 kinase [18] as well as sorafenib [19] and sunitinib [20] sensitive kinases.

Eryptosis is triggered by a large number of xenobiotics [9, 20-46] and excessive eryptosis contributes to the pathophysiology of several diseases [9] including diabetes [9, 13, 47], renal insufficiency [9], hemolytic uremic syndrome [9], sepsis [9], malaria [9], sickle cell disease [9], Wilson's disease [9], iron deficiency [9], malignancy [48], phosphate depletion [9], and metabolic syndrome [42].

The present study explored whether treatment of human erythrocytes with mitotane influenced $[Ca^{2+}]_i$, cell volume and phosphatidylserine abundance at the cell surface. As a result, mitotane significantly increased $[Ca^{2+}]_i$ and significantly enhanced the phosphatidylserine abundance at the erythrocyte surface but did not significantly decrease erythrocyte volume.

Materials and Methods

Erythrocytes, solutions and chemicals

Leukocyte depleted erythrocytes were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003V). Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N 2 hydroxyethylpiperazine N 2 ethanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$; pH 7.4 at 37°C for 24 and 48 h. Where indicated, erythrocytes were exposed to mitotane (Sigma-Aldrich, Germany) at the indicated concentrations. The substance was solved in up to 1.5 µl/ml DMSO. In Ca^{2+} free Ringer solution, 1 mM $CaCl_2$ was substituted by 1 mM glycol bis(2 aminoethylether) N,N,N',N' tetraacetic acid (EGTA).

FACS analysis of annexin V binding and forward scatter

After incubation under the respective experimental condition, 50 µl cell suspension was centrifuged (3min, 1600rpm, 23°C). Ringer solution containing 5 mM $CaCl_2$ was added to the erythrocytes 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, i.e. the scatter of the light in line with the light beam. The FSC is a function of cell volume [49]. Annexin V fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

Measurement of intracellular Ca^{2+}

After incubation 50µl cell suspension was centrifuged (3min, 1600rpm, 23°C). Erythrocytes were loaded with Fluo 3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo

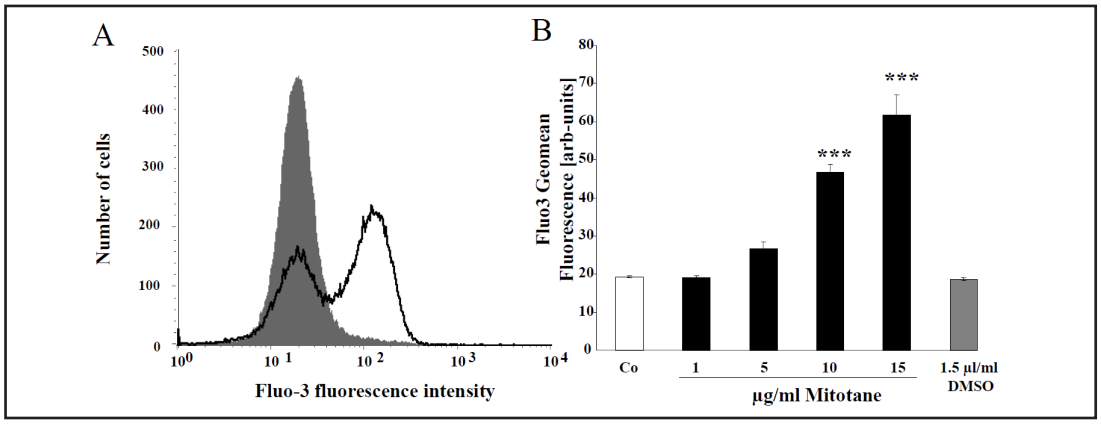

Fig. 1. Effect of mitotane on erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 24 h to Ringer solution without (grey shadow) and with (black line) presence of 15 µg/ml mitotane. B. Arithmetic means ± SEM (n = 5) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 24 h to Ringer solution without (white bar) or with mitotane (1 – 15 µg/ml, black bars) or the solvent DMSO (1.5 µl/ml, grey bars). ***($p<0.001$) indicate significant difference from the absence of mitotane (ANOVA).

3/AM. The cells were incubated at 37°C for 30 min and washed 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 at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

Measurement of hemolysis

For the determination of hemolysis the samples were centrifuged (3 min at 400 g, room temperature) after incubation, and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

Statistics

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

Results

The present study explored whether mitotane stimulates eryptosis, the suicidal death of erythrocytes. Stimulators of eryptosis include increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). Accordingly, the effect of mitotane on $[Ca^{2+}]_i$ has been estimated utilizing Fluo3 fluorescence. Erythrocytes were incubated in Ringer solution without or with mitotane (1 – 15 µg/ml), loaded with Fluo3 AM and Fluo3 fluorescence quantified by FACS analysis. As shown in Fig. 1, a 24 hours exposure of human erythrocytes to mitotane resulted in an increase of Fluo3 fluorescence, an effect reaching statistical significance at 10 µg/ml mitotane concentration. Thus, mitotane treatment was followed by increase of $[Ca^{2+}]_i$ in human erythrocytes. Close inspection of the histogram reveals that mitotane increases Fluo3 fluorescence in the major portion of erythrocytes but does not modify Fluo3 fluorescence in a minor portion of the erythrocytes. As a result, the histogram shows two peaks of Fluo3 fluorescence in erythrocytes following mitotane treatment.

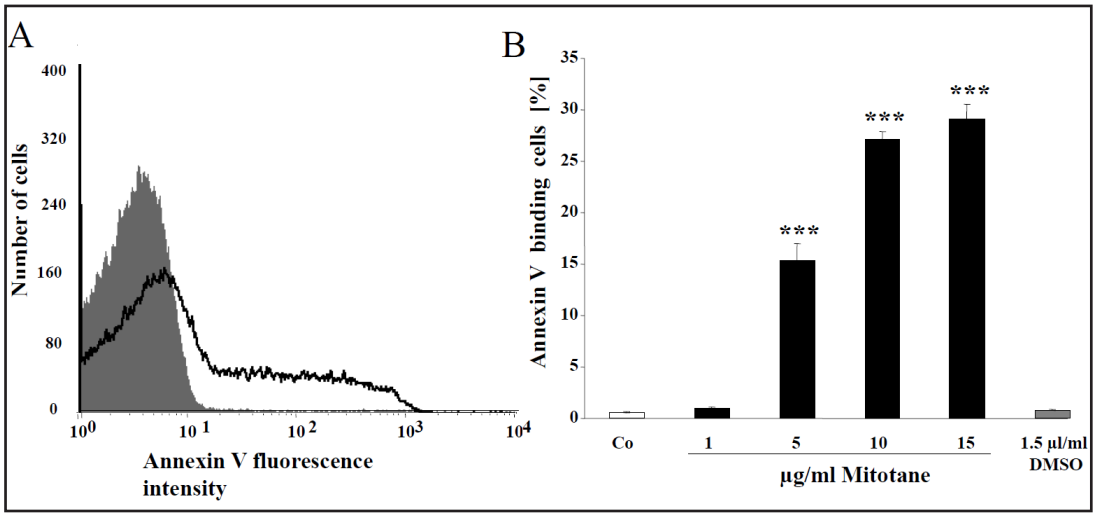

Fig. 2. Effect of mitotane on phosphatidylserine exposure. A. Original histogram of annexin V binding of erythrocytes following exposure for 24 h to Ringer solution without (grey shadow) and with (black line) presence of 15 µg/ml mitotane. B. Arithmetic means ± SEM (n = 5) of erythrocyte annexin V binding following incubation for 24 h to Ringer solution without (white bar) or with mitotane (1 – 15 µg/ml, black bars) or the solvent DMSO (1.5 µl/ml, grey bars). ***(p<0.001) indicate significant differences from the absence of mitotane (ANOVA).

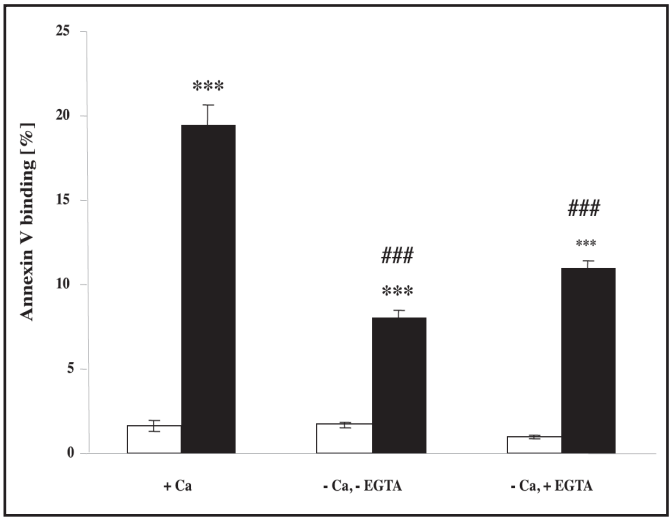

Fig. 3. Effect of Ca^{2+} withdrawal and addition of EGTA on mitotane induced annexin V binding. Arithmetic means ± SEM (n = 6) of the percentage of annexin V binding erythrocytes after a 24 h treatment with Ringer solution without (white bars) or with mitotane (15 µg/ml black bars) in the presence of Ca^{2+} (left bars) or absence of Ca^{2+} in the absence (middle bars) and presence (right bars) of 1 mM EGTA. *(p<0.05),***(p<0.001) indicate significant difference from the absence of mitotane (ANOVA), ###(p<0.001) indicates significant difference from the respective values in the presence of Ca^{2+}.

An increase of $[Ca^{2+}]_i$ has previously been shown to trigger cell membrane scrambling with appearance of phosphatidsylserine at the cell surface. To identify phosphatidylserine exposing erythrocytes, phosphatidylserine abundance at the cell surface was estimated utilizing FITC-labelled annexin V, which was detected in FACS analysis. As illustrated in Fig. 2, a 24 hours mitotane treatment increased the percentage of annexin V binding erythrocytes, an effect reaching statistical significance at 5 µg/ml mitotane concentration. Thus, mitotane treatment triggered erythrocyte cell membrane scrambling.

Additional experiments explored whether the cell membrane scrambling following mitotane treatment was in part or fully dependent on Ca^{2+} entry from the extracellular space. Erythrocytes were exposed to 1 - 15 µg/ml mitotane for 24 hours in either the presence of 1 mM extracellular Ca^{2+} or in the absence of extracellular Ca^{2+} with either presence or absence of the Ca^{2+} chelator EGTA (1 mM). Following mitotane treatment (15 µg/ml), Fluo3 fluorescence reflecting $[Ca^{2+}]_i$ increased from 19.4 ± 0.7 to 29.5 ± 2.1 arbitrary units (n = 5) in the presence of extracellular Ca^{2+} but was in the absence of extracellular Ca^{2+} not significantly different

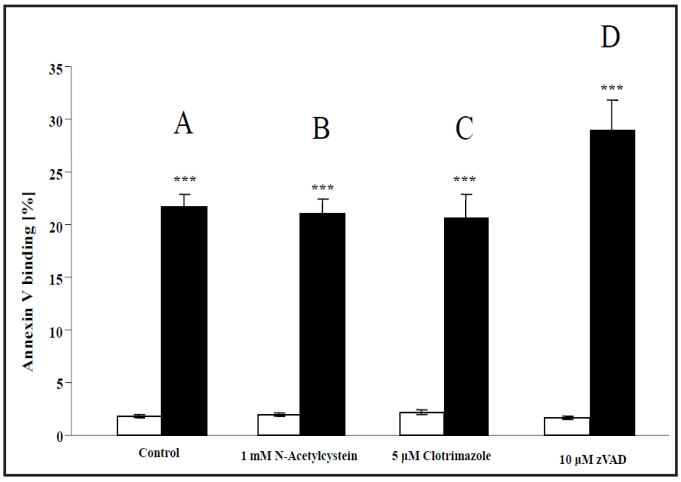

Fig. 4. Effect of K^+ channel inhibitor clotrimazole, of antioxidant N-acetyl-cysteine and of pancaspase inhibitor zVAD on mitotane induced annexin V binding. Arithmetic means ± SEM (n = 6) of the percentage of annexin V binding erythrocytes after a 24 h treatment with Ringer solution without (white bars) or with 15 µg/ml mitotane (black bars) in the absence (A) or presence of 1 mM N-acetyl-cysteine (B), of 5 µM clotrimazole (C), or of 10 µM zVAD (D), respectively.***(p<0.001) indicate significant difference from the absence of mitotane (ANOVA).

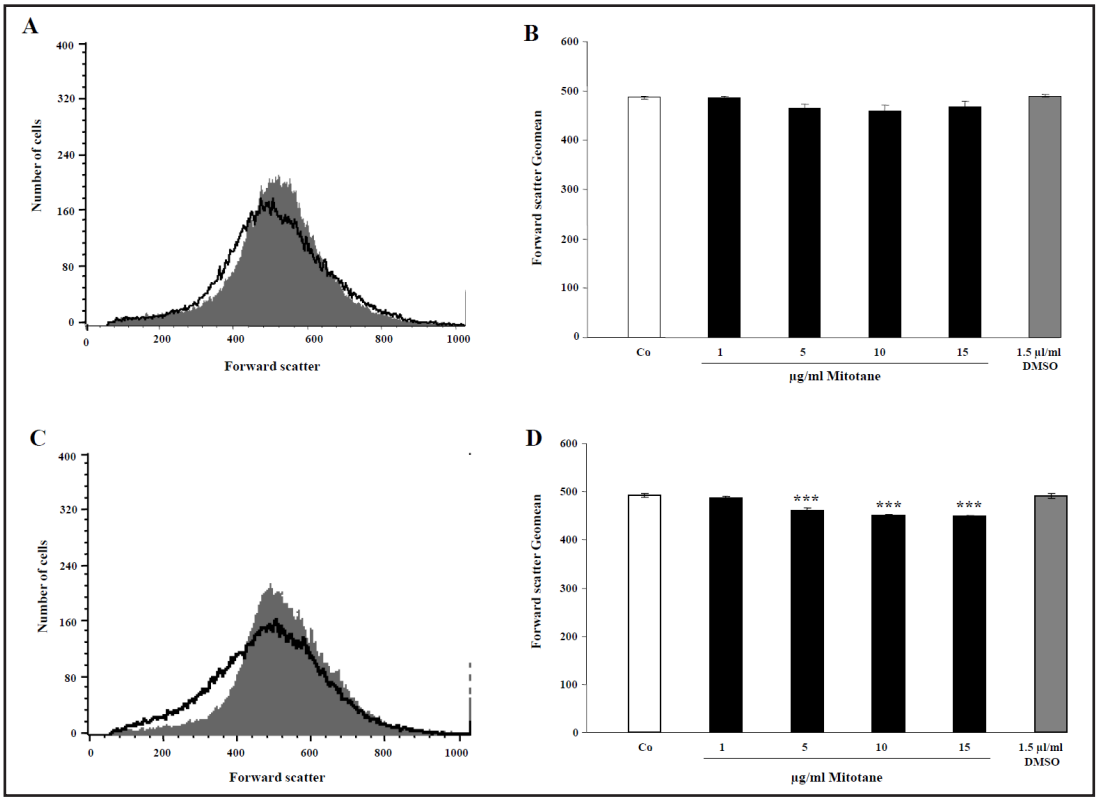

Fig. 5. Effect of mitotane on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 24 h to Ringer solution without (grey shadow) and with (black line) presence of 15 µg/ml mitotane. B. Arithmetic means ± SEM (n = 5) of the normalized erythrocyte forward scatter (FSC) following exposure for 24 h to Ringer solution without (white bar) or with mitotane (1 – 15 µg/ml, black bars) or the solvent DMSO (1.5 µl/ml, grey bars). C. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (grey shadow) and with (black line) presence of 15 µg/ml mitotane. D. Arithmetic means ± SEM (n = 5) of the normalized erythrocyte forward scatter (FSC) following exposure for 48 h to Ringer solution without (white bar) or with mitotane (1 – 15 µg/ml, black bars) or the solvent DMSO (1.5 µl/ml, grey bars). ***(p<0.001) indicate significant difference from the absence of mitotane (ANOVA).

between untreated (20.0 ± 0.9 arbitrary units, n = 5) and mitotane (15 µg/ml) treated (24.3 ± 0.2 arbitrary units (n = 5) erythrocytes. As illustrated in Fig. 3, removal of extracellular

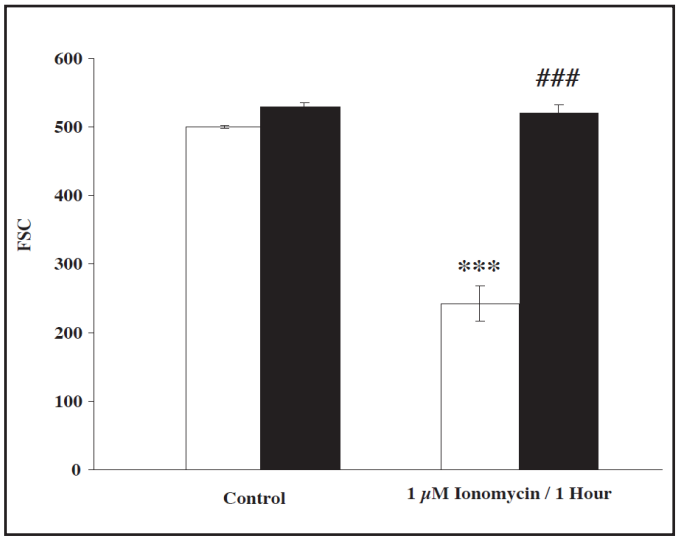

Fig. 6. Effect of Ca^{2+} ionophore ionomycin on forward scatter in the absence and presence of mitotane. Arithmetic means ± SEM (n = 4) of the normalized erythrocyte forward scatter (FSC) after a 1 hour treatment with Ringer solution without (white bars) or with 15 μg/ml mitotane (black bars) in the absence (left bars) or in the presence of 1μM ionomycin (right bars). ***(p<0.001) indicate significant difference from the absence of ionomycin (ANOVA), ###(p<0.001) indicates significant difference from the respective values in the absence of mitotane.

Ca^{2+} significantly blunted the effect of mitotane on annexin V binding irrespective of the presence or absence of EGTA. However, even in the nominal absence of extracellular Ca^{2+} the percentage annexin V binding erythrocytes was still significantly increased by mitotane treatment (Fig. 3). Accordingly, the effect of mitotane on cell membrane scambling was in part but not fully dependent on the presence of extracellular Ca^{2+}.

In search for additional mechanisms triggering cell membrane scrambling following mitotane treatment of erythrocytes, mitotane was applied in the presence and absence of K^+ channel blocker clotrimazole, of caspase inhibitor zVAD and of antioxidant N-acetyl-cystein. As illustrated in Fig. 4, mitotane treatment increased the percentage of annexin V binding cells to a similar extent in the absence and presence of N-acetyl-cysteine (1 mM), clotrimazole (5 μM) or of zVAD (10 μM). The percentage of annexin V binding cells following treatment with mitotane even tended to be higher in the prescence than in the absence of zVAD (Fig. 4), a difference, however, not reaching statistical significance. Clotrimazole has previously been shown to inhibit lead induced eryptosis [50] and N-acetyl-cysteine has previously been shown to inhibit Bay 11-7082 and parthenolide induced eryptosis [28]. Additional experiments were performed to test whether zVAD (10 μM) was effective in another type of eryptosis. To this end erythrocytes were exposed for 48 hours to Ringer or eryptosis inducing kinase inhibitor sunitinib [20] with or without the additional presence of zVAD. As a result, sunitinib (20 μM) increased the percentage annexin V binding cells from 1.5 ± 0.2 to 37.5 ± 3.4 % (n = 6) in the absence and from 1.3 ± 0.3 to 21.7 ± 4.3 % (n = 6) in the presence of zVAD (10 μM). Thus, zVAD blunted the sunitinib induced eryptosis.

An increase of $[Ca^{2+}]_i$ is further expected to activate Ca^{2+} sensitive K^+ channels leading to cellular efflux of KCl together with osmotically obliged water and thus to cell shrinkage. In order to estimate erythrocyte volume, forward scatter was determined in FACS analysis. As illustrated in Fig. 5, mitotane treatment tended to decrease forward scatter within 24 hours and significantly decreased forward scatter within 48 hours.

The lack of erythrocyte shrinkage despite the increase of $[Ca^{2+}]_i$ may have resulted from an inhibitory effect of mitotane on the Ca^{2+} sensitive K^+ channels and/or the Cl^- channels required for the parallel Cl^- efflux. If mitotane was inhibiting those channels, then mitotane treatment would interfere with the erythrocyte shrinkage following increase of $[Ca^{2+}]_i$ upon treatment of erythrocytes with the Ca^{2+} ionophore ionomycin. Thus, forward scatter was determined prior to and 30 minutes following treatment with 1 μM ionomycin in the presence and absence of mitotane. Ionomycin increased the Fluo3 fluorescence reflecting $[Ca^{2+}]_i$ from 20.4 ± 0.2_ to 75.6 ± 6.4 arbitrary units (n = 5) in the absence of mitotane and from 25.1 ± 2.7 to 110.3 ± 6.6 arbitrary units (n = 5) in the presence of mitotane (15 μg/ml).

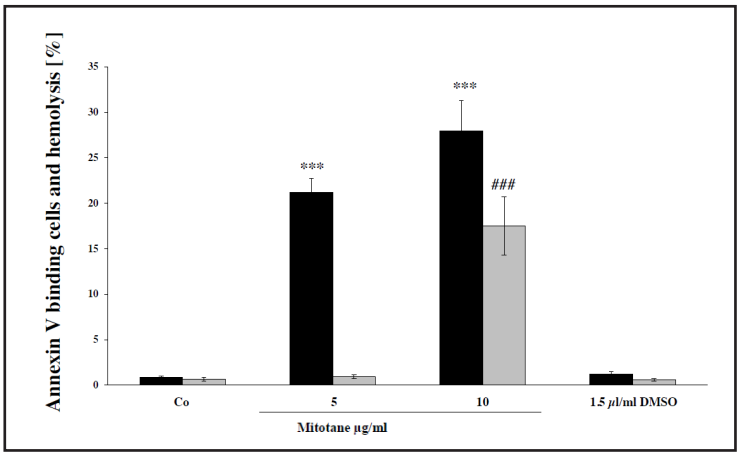

Fig. 7. Effect of short mitotane exposure on annexin V binding and hemolysis. Arithmetic means ± SEM (n = 5) of the percentage of annexin V binding (black bars) or hemolysed (grey bars) erythrocytes following incubation for 2.5 hours to Ringer solution without (Co = control) or with mitotane (5 – 10 µg/ml) or the solvent DMSO (1.5 µl/ml, right bars). ***(p<0.001) indicate significant differences from the absence of mitotane (ANOVA).

As shown in Fig. 6 ionomycin treatment was followed by a sharp decrease of forward scatter in the absence, but not in the presence of mitotane (15 µg/ml).

The inability of mitotane treated erythrocytes to decrease their volume upon increase of $[Ca^{2+}]_i$ may enhance their susceptibility to hemolysis. As illustrated in Fig. 7, the percentage of hemolysed erythrocytes was low following treatment of erythrocytes for 2.5 hours with 5 µg/ml mitotane but increased to almost the same value following a 2.5 hours treatment with 10 µg/ml mitotane. Following a 24 hours exposure to mitotane the percentage of hemolysed erythrocytes amounted to (n = 5 each) 0.5 ± 0.2 % (absence of mitotane), 0.4 ± 0.2 % (at 1 µg/ml mitotane), 20.0 ± 2.6% (at 5 µg/ml mitotane), 56.7 ± 2.5 % (at 10 µg/ml mitotane) and 62.8 ± 2.8 % (at 15 µg/ml mitotane). The hemolysis reached statistical significance at 5 µg/ml mitotane concentration.

Discussion

The present study reveals that mitotane triggers cell membrane scrambling of erythrocytes. Mitotane treatment of erythrocytes drawn from healthy volunteers is followed by breakdown of phosphatidylserine asymmetry of the cell membrane, a hallmark of eryptosis. The concentrations required to stimulate eryptosis are in the range of the reported therapeutic threshold concentrations of 14 - 20 µg/ml and still below toxic mitotane concentrations exceeding 30 µg/ml [51].

The breakdown of phosphatidylserine asymmetry of the erythrocyte cell membrane following mitotane treatment was presumably in part due to an increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). The phosphatidylserine abundance at the surface of mitotane treated erythrocytes was significantly blunted in the absence of extracellular Ca^{2+}. An increase of $[Ca^{2+}]_i$ is well known to stimulate cell membrane scrambling with phosphatidylserine translocation from the inner leaflet of the cell membrane to the outer leaflet of the cell membrane [9]. The channels mediating Ca^{2+} entry are Ca^{2+} permeable non selective cation channels involving the transient receptor potential channel TRPC6 [9]. The Ca^{2+} permeable erythrocyte cation channels are stimulated by oxidative stress [9]. The lacking inhibitory effect of antioxidant N-acetyl-cystein suggests, however, that mitotane does not activate the channels by inducing oxidatitve stress.

Notably, a minor portion of erythrocytes appears to maintain the Fluo3 fluorescence following mitotane treatment, a result pointing to heterogeneity in mitotane sensitivity of the erythrocyte population. Earlier experiments revealed that sensitivity to triggers of eryptosis may be affected by erythrocyte age [27].

The mitotane sensitivity of $[Ca^{2+}]_i$ and the blunted effect of mitotane on annexin V binding in the absence of extracellular Ca^{2+} clearly indicate that mitotane is at least partially

effective by triggering Ca^{2+} entry. The search for further mechanisms involved were not successful. Mitotane induced eryptosis was not significantly modified by K^+ channel blocker clotrimazole, antioxidant N-acetyl-cysteine and caspase inhibitor zVAD. Thus, the effect of mitotane does apparently not require activation of K^+ channels, oxidative stress or activation of caspases. In theory, mitotane could influence some of the kinases participating in the regulation of eryptosis, such as AMPK [9], G kinase [9], JAK3 [14], casein kinase [15, 16], p38 kinase [17], PAK2 kinase [18] or sorafenib [19] and sunitinib [20] sensitive kinases. In any case, the effect of mitotane on cell membrane scrambling is in large part due to Ca^{2+} entry.

An increase of $[Ca^{2+}]_i$ was expected to activate Ca^{2+} sensitive K^+ channels [10, 52] leading to cell membrane hyperpolarization. The increased electrical driving force was expected to drive Cl^- efflux with cellular efflux of KCl and osmotically obliged water thus resulting in erythrocyte shrinkage [9]. However, despite the increase of $[Ca^{2+}]_i$, mitotane treatment was not followed by appreciable cell shrinkage. Moreover, mitotane abrogated the cell shrinkage following increase of $[Ca^{2+}]_i$ with Ca^{2+} ionophore ionomycin. Presumably, mitotane inhibits the Ca^{2+} sensitive K^+ channels and/or the Cl^- channels in the erythrocyte cell membane. Entry of Na^+ through the unselective cation channels and simultaneous inhibition of Ca^{2+} sensitive K^+ channels may foster cell swelling, which could eventually result in disruption of the erythrocyte membrane and thus hemolysis.

The stimulation of cell membrane scrambling by mitotane may lead to anemia. Phosphatidylserine exposing erythrocytes are rapidly removed from circulating blood [9]. If the clearance of phosphatidylserine exposing erythrocytes is not matched by formation of new erythrocytes, anemia develops [9]. Knowledge about the effect of mitotane on anemia is scarce [7, 8]. It must be kept in mind, that the drug is used in malignancy [1-3], a disorder causing anemia [53]. Thus, during mitotane treatment the eryptotic effects of mitotane may be outweighed by the effects of decreasing tumor mass.

Phosphatidylserine exposing erythrocytes may further bind to endothelial CXCL16/SR PSO [54] and adhere to the vascular wall thus compromizing microcirculation [54-59]. Phosphatidylserine exposure further fosters blood clotting and thus predisposes to development of thrombosis [55, 60, 61].

In conclusion, exposure of human erythrocytes to mitotane triggers Ca^{2+} entry with subsequent stimulation of cell membrane scrambling. Mitotane does, however, not decrease cell volume.

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Disclosure Statement

The authors declare that they have no potential conflict of interest.

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