Original Paper

Triggering of Suicidal Erythrocyte Death by Bexarotene

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Key Words

Phosphatidylserine • Eryptosis • D4476 • Casein kinase • Oxidative stress • Calcium

Abstract

Background/Aims: The retinoid X receptor agonist bexarotene is utilized for the treatment of cutaneous T-cell lymphoma and is effective in several further malignancies. The substance counteracts tumor growth in part by triggering suicidal death or apoptosis of tumor cells. Side effects of bexarotene treatment include anemia. Theoretically, bexarotene induced anemia could be secondary to stimulation of suicidal erythrocyte death or eryptosis, characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Signaling potentially stimulating eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), induction of oxidative stress, increase of ceramide abundance, as well as activation of staurosporine sensitive protein kinase C, SB203580 sensitive p38 kinase, D4476 sensitive casein kinase 1, and zVAD sensitive caspases. The present study explored, whether bexarotene induces eryptosis and, if so, whether its effect involves Ca^{2+} entry, oxidative stress, ceramide, kinases and/or caspases. **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 48 hours exposure of human erythrocytes to bexarotene ($\geq 0.4 \mu\text{g/ml}$) significantly increased the percentage of annexin-V-binding cells without significantly modifying forward scatter. Bexarotene significantly increased Fluo3-fluorescence and DCFDA fluorescence. Bexarotene tended to increase ceramide abundance, an effect, however, not reaching statistical significance. The effect of bexarotene on annexin-V-binding was significantly blunted by removal of extracellular Ca^{2+} and by addition of D4476 ($10 \mu\text{M}$), but not by addition of staurosporine ($1 \mu\text{M}$), SB203580 ($2 \mu\text{M}$), or zVAD ($10 \mu\text{M}$). **Conclusions:** Bexarotene triggers phospholipid scrambling of the erythrocyte cell membrane, an effect at least in part due to Ca^{2+} entry, oxidative stress, and activation of D4476 sensitive casein kinase.

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Introduction

Bexarotene, a synthetic analog (4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethynyl]benzoic acid) of retinoids [1, 2], selectively binds to and activates the retinoid X receptor (RXRs) subfamilies [1-8], thus inhibiting tumor growth both, *in vitro* and *in vivo* [1]. The substance is used for the treatment of cutaneous T-cell lymphoma (CTCL) [3, 5, 6, 8-28], and has been shown to be effective in other malignancies, such as non-small cell lung cancer [16, 29, 30], breast cancer [16, 31-33], and – in animals – colon cancer [34], breast cancer [35, 36] and lung cancer [37]. However, bexarotene treatment may trigger extracutaneous lymphoma [38]. Moreover, bexarotene has been used in psoriasis [39].

In the brain, bexarotene has been shown to provide neuroprotection in traumatic brain injury [40] by inhibiting neuronal apoptosis, an effect requiring Apo protein E [40]. Along those lines, bexarotene has been shown to reverse neurodegeneration, improve cognition, and decrease the levels of amyloid- β in transgenic mice expressing familial Alzheimer disease mutations [41].

Bexarotene is at least in part effective by blocking cell cycle progression [4, 42], inducing apoptosis [4-6, 8, 24, 27, 28, 34, 35, 37, 43-48], fostering differentiation [4, 35, 39], preventing multidrug resistance [4, 33], inhibiting angiogenesis [4], and counteracting metastasis [4]. Signaling triggered by bexarotene includes ceramide formation [31], activation of the p53/p73 pathway [42], and mitochondrial release of cytochrome C [48].

Common side effects of systemic bexarotene treatment include hyperlipidemia [6, 14, 16, 17, 49], hypothyroidism [6, 14, 16, 20, 49], headache [6], xeroderma [16], leukopenia or neutropenia [49], nasopharyngitis [49], and anemia [49].

In view of the stimulating effect of bexarotene on suicidal death of nucleated cells [4-6, 8, 24, 27, 28, 34, 35, 43-48], the anemia could result from suicidal death of erythrocytes or eryptosis [50].

The hallmark of eryptosis is cell membrane scrambling with phosphatidylserine translocation to the cell surface [50]. The cell membrane scrambling is usually paralleled by cell shrinkage [51]. Signaling involved in the triggering of eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [50], ceramide [52], caspases [50, 53, 54], stimulated activity of casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, p38 kinase and PAK2 kinase [50], as well as impaired activity of AMP activated kinase AMPK, cGMP-dependent protein kinase, and sorafenib/sunitinib sensitive kinases [50]. Stimulators of eryptosis include hyperosmotic shock [50], oxidative stress [50], energy depletion [50], or exposure to a wide variety of xenobiotics [50, 55-99]. Enhanced eryptosis is observed in a variety of clinical conditions including dehydration [100], hyperphosphatemia [101], chronic kidney disease (CKD) [102-105], hemolytic-uremic syndrome [106], diabetes [107], hepatic failure [56], malignancy [50], sepsis [108], sickle-cell disease [50], beta-thalassemia [50], Hb-C and G6PD-deficiency [50], as well as Wilsons disease [109].

The present study explored, whether bexarotene is able to trigger eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to bexarotene and phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$, 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 48 hours. Where indicated, erythrocytes were exposed for 48 hours to bexarotene (MedChem

Express, Princeton, USA). In order to estimate the impact of Ca^{2+} entry on bexarotene induced eryptosis, erythrocytes were exposed to bexarotene 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 48 hours to a combination of bexarotene and p38 kinase inhibitor SB203580 (Tocris bioscience, Bristol, UK), protein kinase C inhibitor staurosporine (Enzo Life Sciences, Lörrach, Germany) or casein kinase inhibitor D4476 (Tocris Bioscience, Bristol, UK). The impact of caspases was elucidated utilizing the pancaspase inhibitor zVAD (Enzo Life Sciences, Lörrach, Germany).

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 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 15 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 bexarotene 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 150 μl 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 μM . 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 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). 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 $\mu\text{g}/\text{ml}$ 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 bexarotene stimulates eryptosis, the suicidal erythrocyte death. The most important hallmark of eryptosis is phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface. Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding to phosphatidylserine, as determined by flow cytometry. Annexin-V-binding was analysed following an incubation of the erythrocytes for 48 hours in Ringer solution without or with bexarotene (0.2 – 0.6 µg/ml). As illustrated in Fig. 1, a 48 hours treatment with bexarotene increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 0.4 µg/ml bexarotene.

A second hallmark of eryptosis is erythrocyte shrinkage. As a measure of erythrocyte volume forward scatter was determined utilizing flow cytometry. The measurements were performed after incubation for 48 hours in Ringer solution without or with bexarotene (0.2 – 0.6 µg/ml). As a result, the erythrocyte forward scatter was similar following exposure in the absence of bexarotene (496.5 ± 8.8 a.u., $n = 14$) and in the presence of 0.2 µg/ml bexarotene (482.5 ± 7.6 a.u., $n = 14$), 0.4 µg/ml bexarotene (484.4 ± 16.6 a.u., $n = 14$) and 0.6 µg/ml bexarotene (480.0 ± 14.9 a.u., $n = 14$). Accordingly, bexarotene did not significantly modify erythrocyte forward scatter.

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 48 hours incubation in Ringer solution without or with bexarotene (0.2 – 0.6 µg/ml). As illustrated in

Fig. 1. Effect of bexarotene on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 0.6 µg/ml bexarotene. B. Arithmetic means $\pm$ SEM ($n = 14$) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) bexarotene (0.2 – 0.6 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). ***($p < 0.001$) indicates significant difference from the absence of bexarotene (ANOVA).

Fig. 2. Effect of bexarotene on cytosolic Ca^{2+} activity. A. Original histogram of Fluo3 fluorescence of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 0.6 µg/ml bexarotene. B. Arithmetic means $\pm$ SEM ($n = 14$) of erythrocyte Fluo3 fluorescence following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) bexarotene (0.2 – 0.6 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). ***($p < 0.001$) indicates significant difference from the absence of bexarotene (ANOVA).

Fig. 2, a 48 hours exposure to bexarotene increased the Fluo3 fluorescence, an effect reaching statistical significance at 0.4 $\mu\text{g/ml}$ bexarotene.

In order to test whether bexarotene-induced translocation of phosphatidylserine to the outside required entry of extracellular Ca^{2+} , erythrocytes were incubated for 48 hours in the absence or presence of 0.6 $\mu\text{g/ml}$ bexarotene in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 3, removal of extracellular Ca^{2+} significantly blunted the effect of bexarotene on annexin-V-binding. However, even in the absence of extracellular Ca^{2+} , bexarotene significantly increased the percentage of annexin-V-binding erythrocytes. Thus, the bexarotene-induced cell membrane scrambling was in part but not fully triggered by entry of extracellular Ca^{2+} .

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As illustrated in Fig. 4, a 48 hours exposure to bexarotene (0.6 $\mu\text{g/ml}$) increased the DCFDA fluorescence of erythrocytes. The observations suggest that bexarotene did induce oxidative stress.

A further stimulator of eryptosis is ceramide. Ceramide abundance at the

erythrocyte surface was quantified utilizing specific antibodies. As a result, the ceramide abundance tended to be higher following exposure to 0.6 $\mu\text{g/ml}$ bexarotene (22.0 ± 1.7 a.u., $n = 10$) than following incubation in the absence of bexarotene (18.8 ± 0.6 a.u., $n = 10$), a difference, however, not reaching statistical significance.

Fig. 3. Ca^{2+} sensitivity of bexarotene-induced phosphatidylserine exposure. A, B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) bexarotene (0.6 $\mu\text{g/ml}$) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means $\pm$ SEM ($n = 10$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) bexarotene (0.6 $\mu\text{g/ml}$) 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 bexarotene, ###($p < 0.001$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

Fig. 4. Effect of bexarotene on reactive oxygen species. A. Original histogram of DCFDA fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 0.6 $\mu\text{g/ml}$ bexarotene. B. Arithmetic means $\pm$ SEM ($n = 9$) of DCFDA fluorescence in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bar) bexarotene (0.6 $\mu\text{g/ml}$). ***($p < 0.01$) indicates significant difference from the absence of bexarotene (unpaired t test).

Fig. 5. D4476 sensitivity of bexarotene-induced phosphatidylserine exposure. A, B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) bexarotene (0.6 µg/ml) in the absence (A) and presence (B) of casein kinase inhibitor D4476 (10 µM). C. Arithmetic means $\pm$ SEM (n = 9) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) bexarotene (0.6 µg/ml) in the absence (left bars, -D4476) and presence (right bars, +D4476) of casein kinase inhibitor D4476 (10 µM). *** (p < 0.001) indicates significant difference from the absence of bexarotene, # (p < 0.05) indicates significant difference from the presence of D4476 (10 µM) (ANOVA).

Fig. 6. Effect of bexarotene on hemolysis. Arithmetic means $\pm$ SEM (n = 5) of the percentage of hemolyzed erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) bexarotene (0.2 – 0.6 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). *** (p < 0.001) indicates significant difference from the absence of bexarotene (ANOVA).

To explore, whether the effects of bexarotene involved kinase activity, the influence of bexarotene on annexin-V-binding was tested in the presence of p38 kinase inhibitor SB203580, of protein kinase C inhibitor staurosporine, or of casein kinase 1 inhibitor D4476. As a result, bexarotene (0.6 µg/ml) increased the percentage of phosphatidylserine exposing erythrocytes to similar values following incubation in the absence (from 1.2 ± 0.1 % to 23.1 ± 1.6 %, n = 10) and in the presence (from 1.3 ± 0.1 % to 24.5 ± 4.0 %, n = 10) of SB203580 (2 µM). Bexarotene (0.6 µg/ml) further increased the percentage of phosphatidylserine exposing erythrocytes to similar values following incubation in the absence (from 1.4 ± 0.3 % to 28.4 ± 2.0 %, n = 15) and in the presence (from 2.9 ± 0.3 % to 31.8 ± 2.6 %, n = 15) of staurosporine (1 µM). As illustrated in Fig. 5, D4476 (10 µM) significantly blunted the effect of bexarotene on annexin-V-binding. However, even in the presence of D4476, bexarotene significantly increased the percentage of annexin-V-binding erythrocytes. Accordingly, bexarotene-induced cell membrane scrambling was in part but not fully dependent on D4476 sensitive casein kinase 1.

To test whether the effects of bexarotene involved caspases, the influence of bexarotene on annexin-V-binding was tested in the presence of pancaspase inhibitor zVAD. As a result, bexarotene (0.6 µg/ml) increased the percentage of phosphatidylserine exposing erythrocytes to similar values following incubation in the absence (from 1.3 ± 0.1 % to 18.7 ± 1.1 %, n = 5) and in the presence (from 2.2 ± 0.5 % to 20.6 ± 2.4 %, n = 5) of zVAD (10 µM).

A final series of experiments addressed the influence of bexarotene on hemolysis. The percentage of hemolytic cells was quantified from the hemoglobin concentration in the supernatant. As illustrated in Fig. 6, a 48 hours exposure to bexarotene increased the percentage of hemolytic erythrocytes, an effect reaching statistical significance at 0.4 µg/ml bexarotene.

Discussion

The present observations reveal that bexarotene stimulates cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface, a hallmark of eryptosis, i.e. of suicidal erythrocyte death. The bexarotene concentrations required for the stimulation of eryptosis are well in the range of concentrations (0.2 – 6 µg/ml) encountered in the plasma of patients [110, 111]. The stimulation of eryptosis may thus well explain the anemia following bexarotene treatment [49].

The effect of bexarotene on cell membrane scrambling was paralleled by an increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) and was in large part dependent on Ca^{2+} entry from the extracellular space. Removal of extracellular Ca^{2+} significantly blunted the bexarotene induced eryptosis. However, bexarotene triggered cell membrane scrambling even in the nominal absence of extracellular Ca^{2+} , an observation pointing to the involvement of additional mechanisms contributing to bexarotene induced cell membrane scrambling. Cells could be sensitized for the scrambling effect of Ca^{2+} by ceramide [50]. Bexarotene treatment tended to increase the ceramide abundance, an effect not reaching statistical significance. Bexarotene significantly enhanced the abundance of reactive oxygen species, a well known trigger of eryptosis [50]. Bexarotene is therefore partially effective by inducing oxidative stress. The effect of bexarotene did not require the activity of SB203580 sensitive p38 kinase or of staurosporine sensitive kinases such as protein kinase C. Both kinases participate in the orchestration of eryptosis by other stimuli [50]. The bexarotene induced cell membrane scrambling was slightly, but significantly blunted by casein kinase 1 inhibitor D4476 and may involve the casein kinase 1, a known stimulator of eryptosis [50].

The effect of bexarotene on cell membrane scrambling was not paralleled by appreciable erythrocyte shrinkage. This observation is surprising in view of the marked increase of $[\text{Ca}^{2+}]_i$ which were expected to activate Ca^{2+} sensitive K^+ channels leading to K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water. The underlying mechanism accounting for the failure of bexarotene treated erythrocytes to shrink remained elusive. Potential mechanisms include an inhibitory effect on the Na^+/K^+ ATPase with decrease of cellular K^+ concentration and thus failure to hyperpolarize following activation of K^+ channels. Alternatively erythrocyte shrinkage could be prevented by inhibition of K^+ or Cl^- channels.

Inability to release K^+ and Cl^- through the respective ion channels would not only be expected to disrupt cell shrinkage but may actually lead to cell swelling. Excessive cell swelling may be followed by disruption of the cell membrane, a key event of hemolysis. *In vivo* eryptosis aims to accomplish clearance of defective erythrocytes from circulating blood prior to hemolysis [50]. The hemoglobin released following hemolysis may otherwise pass the renal glomerular filter, precipitate in the acidic lumen of renal tubules, occlude nephrons and may thus lead to renal failure [112].

The clearance of phosphatidylserine exposing erythrocytes may lead to anemia as soon as the loss of erythrocytes surpasses the formation of new erythrocytes by erythropoiesis [50]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [113], stimulate blood clotting and trigger thrombosis [114-116], and may thus interfere with microcirculation [52, 114, 117-120].

In conclusion, bexarotene triggers erythrocyte cell membrane scrambling, an effect in part due to Ca^{2+} entry, oxidative stress and activation of D4476 sensitive casein kinase 1.

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Disclosure Statement

All authors declare that there are no conflicts of interest.

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