Original Paper

Effect of Bexarotene on Platelet Activation and Apoptosis

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Key Words

Thrombin • Collagen related peptide • Platelet activation • Degranulation • Integrin • Cytosolic Ca²⁺ concentration • Caspase • Phosphatidylserine

Abstract

Background/Aims: The retinoid X receptor (RXRs) stimulator Bexarotene ((4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethynyl] benzoic acid) is used for the treatment of several malignancies. Bexarotene is at least in part effective by stimulation of apoptosis of tumor cells. Moreover, Bexarotene triggers eryptosis, the suicidal death of erythrocytes. Similar to erythrocytes, blood platelets lack nuclei but are nevertheless able to enter an apoptosis-like phenotype, characterized by caspase activation, cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface. Platelet apoptosis is triggered by increase of cytosolic Ca²⁺-activity ([Ca²⁺]_i), which further leads to degranulation and integrin activation. Platelet activation and apoptosis could be elicited by thrombin or collagen related peptide (CRP). The present study explored whether treatment of platelets with bexarotene modifies platelet activation and apoptosis following exposure to thrombin or CRP. **Methods:** Platelets isolated from wild-type mice were exposed for 30 minutes to bexarotene (6 µg/ml) without or with an additional treatment with thrombin (0.01 U/ml) or CRP (2 µg/ml or 5 µg/ml). Flow cytometry was employed to estimate cytosolic Ca²⁺-activity ([Ca²⁺]_i) from Fluo-3 fluorescence, platelet degranulation from P-selectin abundance, integrin activation from α_{IIb}β₃ integrin abundance, caspase activity utilizing an Active Caspase-3 Staining kit, phosphatidylserine abundance from annexin-V-binding, and relative platelet volume from forward scatter. **Results:** In the absence of thrombin or CRP, the administration of bexarotene slightly but significantly increased [Ca²⁺]_i, but did not significantly modify P-selectin abundance, activated α_{IIb}β₃ integrin, annexin-V-binding, cell volume, or caspase activity. Exposure of platelets to thrombin or CRP was followed by significant increase of [Ca²⁺]_i, P-selectin abundance, active α_{IIb}β₃ integrin, annexin-V-binding, and caspase activity. The effects of thrombin on [Ca²⁺]_i, annexin-V-binding, cell volume, and caspase activity as well as the effects of CRP on [Ca²⁺]_i, P-selectin abundance, activated α_{IIb}β₃ integrin, annexin-V-binding, cell volume, and caspase activity were significantly augmented in the presence of bexarotene. **Conclusions:** Bexarotene sensitizes blood platelets for thrombin and/or CRP induced activation and apoptosis.

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Introduction

Bexarotene ([4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethynyl] benzoic acid) [1, 2], a stimulator of retinoid X receptors (RXRs) [1-8], has previously been shown to counteract tumor growth [2]. Bexarotene is thus used for the treatment of several malignancies such as cutaneous T-cell lymphoma (CTCL) [4, 6-29], non-small cell lung cancer [13, 30, 31], and breast cancer [13, 32-34]. In animals, bexarotene proved to be effective in colon cancer [35], breast cancer [8, 36], and lung cancer [37]. Bexarotene has further been used in psoriasis [38]. Bexarotene may confer neuroprotection in traumatic brain injury [39] and reverse neurodegeneration [40].

The effects of bexarotene on tumor growth result at least in part from stimulation of apoptosis [4-6, 8, 11, 18, 29, 35-37, 41-46]. Moreover, bexarotene triggers eryptosis, the suicidal erythrocyte death [47].

Similar to nucleated cells and erythrocytes, blood platelets may enter an apoptosis-like phenotype involving caspase activation, cell shrinkage, and cell membrane scrambling with phosphatidylserine translocation to the cell surface [48, 49]. Platelets participate in the orchestration of primary hemostasis following vascular injury and contribute to the pathophysiology of arterial thrombosis, vascular inflammation and atherogenesis [50, 51]. Platelets are activated by an increase of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) [52], which may result from Ca^{2+} release from intracellular stores [53] and subsequent store operated Ca^{2+} entry (SOCE) due to activation of Ca^{2+} release-activated channel Orai1 in the plasma membrane [52-55].

To the best of our knowledge, nothing is known about an effect of bexarotene on platelet apoptosis. The present study thus explored, whether bexarotene influences platelet activation and/or apoptosis.

Materials and Methods

Mice

All animal experiments were conducted according to the German law for the welfare of animals and were approved by the authorities of the state of Baden-Württemberg. Experiments were performed with blood platelets isolated from wild type mice. The mice had free access to water and control chow (Ssniff, Soest, Germany).

Preparation of mouse platelets

Platelets were obtained from 10- to 12-week-old mice of either sex. The mice were anesthetized and 800 μl blood was drawn from the retro-orbital plexus into tubes with 200 μl acid-citrate-dextrose buffer before the mice were sacrificed [56]. Platelet rich plasma (PRP) was obtained by centrifugation at 260 g for 5 minutes. Afterwards, PRP was centrifuged at 640 g for 5 minutes to pellet the platelets. Where necessary apyrase (0.02 U/ml; Sigma-Aldrich) and prostaglandin I_2 (0.5 μM ; Calbiochem) were added to the PRP to prevent activation of platelets during isolation [57]. After two washing steps, the pellet of washed platelets was resuspended in modified Tyrode-HEPES buffer (pH 7.4, supplemented with 1 mM CaCl_2). Where indicated, thrombin (Roche, Basel, Switzerland) or collagen related peptide (kindly provided by R. Farndale, University of Cambridge, Cambridge, UK) was added at the indicated concentrations.

Cytosolic calcium

For the measurement of the cytosolic Ca^{2+} concentration, the platelet preparation was washed once in Tyrode buffer (pH 7.4), stained with 3 μM Fluo-3AM (Biotinium, USA) in the same buffer and incubated at 37°C for 30 minutes. Following the indicated experimental treatment, relative fluorescence was measured utilizing a BD FACS Calibur (BD Biosciences, Heidelberg, Germany) [58, 59].

P-selectin and activated integrin abundance

Fluorophore-labeled antibodies were utilized for the detection of P-selectin expression (Wug.E9-FITC) [60] and the active form of $\alpha_{\text{IIb}}\beta_3$ integrin (JON/A-PE) [61]. Washed mouse platelets (1×10^6) were suspended

in modified Tyrode buffer (pH 7.4) containing 1 mM CaCl_2 and antibodies (1:10 dilution). They were subsequently subjected to the respective treatments for the indicated time periods at room temperature (RT). The reaction was stopped by addition of PBS and the samples were immediately analyzed on a BD FACSCalibur.

Caspase-3 activity

Caspase 3 activity was determined utilizing a CaspGlow Fluorescein Active Caspase-3 Staining kit from BioVision (CA, USA) according to the manufacturer's instruction. Fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 in a BD FACSCalibur (BD Biosciences, USA).

Phosphatidylserine exposure and forward scatter

Phosphatidylserine exposure was determined in platelets with and without 10 minutes thrombin treatment. To this end, the platelet preparation was centrifuged at 660 g for 5 minutes followed by washing once with Tyrode buffer (pH 7.4) with 1 mM CaCl_2 , staining with 1:20 dilution of Annexin-V FITC (Mabtag, Germany) in Tyrode buffer (pH 7.4) with 2 mM CaCl_2 and incubation at 37°C for 30 minutes. Annexin-V binding reflecting surface exposure of phosphatidylserine was evaluated by flow cytometry utilizing a BD FACSCalibur. In parallel, the forward scatter (FSC) of the platelets was determined by flow cytometry as a measure of platelet size [62].

Statistical analysis

Data are provided as means $\pm$ SEM; *n* represents the number of independent experiments. All data were tested for significance using ANOVA with Tukey's test as post-test or unpaired student's *t*-test as appropriate. Results with *p* < 0.05 were considered statistically significant.

Results

The present study explored whether bexarotene triggers activation and apoptosis of blood platelets. To this end, murine platelets were isolated from wild type mice and exposed to thrombin (0.01 U/ml) or collagen related peptide (CRP, 2 $\mu\text{g}/\text{ml}$ or 5 $\mu\text{g}/\text{ml}$) in the absence and presence of bexarotene (6 $\mu\text{g}/\text{ml}$).

Fig. 1. Bexarotene sensitive thrombin- and CRP-induced increase of cytosolic Ca^{2+} concentration. A-C. Original histogram overlays of Fluo-3 fluorescence reflecting cytosolic Ca^{2+} activity in murine platelets (A) without and (B) with a 100 seconds thrombin (0.01 U/ml) treatment or (C) with a 150 seconds CRP (2 $\mu\text{g}/\text{ml}$) treatment without (grey areas) and with (black lines) presence of bexarotene (6 $\mu\text{g}/\text{ml}$, 30 min). D. Arithmetic means $\pm$ SEM (*n* = 6) of Fluo-3 fluorescence reflecting cytosolic

Ca^{2+} activity in murine platelets without (left bars, resting) and with a 100 seconds thrombin (0.01 U/ml) treatment (middle bars) or a 150 seconds CRP (2 $\mu\text{g}/\text{ml}$) treatment (right bars) in the absence (white bars) and presence (black bars) of 6 $\mu\text{g}/\text{ml}$ bexarotene. * (*p* < 0.05), ** (*p* < 0.01), *** (*p* < 0.001) indicates statistically significant difference from absence of bexarotene, ### (*p* < 0.001) indicates statistically significant difference from absence of thrombin and CRP.

Fig. 2. Bexarotene sensitive thrombin- and CRP-induced increase of surface P-selectin. A-C. Original histogram overlays of P-selectin related fluorescence reflecting degranulation of murine platelets (A) without and (B) with a 15 min thrombin (0.01 U/ml) treatment or (C) with a 15 min CRP (2 μ g/ml) treatment without (grey areas) and with (black lines) presence of bexarotene (6 μ g/ml, 30 min). D. Arithmetic means $\pm$ SEM (n = 6) of P-selectin related fluorescence reflecting degranulation of murine platelets without (left bars, resting) and with a 15 min thrombin (0.01 U/ml) treatment (middle bars) or a 15 min CRP (2 μ g/ml) treatment (right bars) in the absence (white bars) and presence (black bars) of 6 μ g/ml bexarotene. **($p < 0.01$) indicates statistically significant difference from absence of bexarotene, ###($p < 0.001$) indicates statistically significant difference from absence of thrombin and CRP.

Fig. 3. Bexarotene sensitive thrombin- and CRP-induced increase of integrin $\alpha_{IIb}\beta_3$ activation. A-C. Original histogram overlays of activated $\alpha_{IIb}\beta_3$ integrin related fluorescence in murine platelets (A) without and (B) with a 15 min thrombin (0.01 U/ml) treatment or (C) with a 15 min CRP (2 μ g/ml) treatment without (grey areas) and with (black lines) presence of bexarotene (6 μ g/ml, 30 min). D. Arithmetic means $\pm$ SEM (n = 6) of activated $\alpha_{IIb}\beta_3$ integrin related fluorescence in murine platelets without (left bars, resting) and with a 15 min thrombin (0.01 U/ml) treatment (middle bars) or a 15 min CRP (2 μ g/ml) treatment (right bars) in the absence (white bars) and presence (black bars) of 6 μ g/ml bexarotene. **($p < 0.01$) indicates statistically significant difference from absence of bexarotene, ###($p < 0.001$) indicates statistically significant difference from absence of thrombin and CRP.

In a first series of experiments, cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined utilizing Fluo-3 fluorescence. Prior to thrombin or CRP treatment, $[Ca^{2+}]_i$ was slightly but significantly increased by bexarotene treatment (Fig 1). Exposure to thrombin or CRP was followed by a significant increase of $[Ca^{2+}]_i$ in platelets, an effect significantly augmented in the presence of bexarotene (Fig. 1).

Platelet degranulation was estimated from the increase of P-selectin abundance at the platelet surface, which was determined utilizing specific antibodies and flow cytometry. As illustrated in Fig. 2, the P-selectin abundance was negligible and not significantly modified by bexarotene treatment at the surface of resting platelets. Thrombin or CRP significantly increased the P-selectin abundance. The effect of CRP was significantly augmented in the presence of bexarotene (Fig. 2).

Similarly, the abundance of active integrin $\alpha_{IIb}\beta_3$ at the surface of resting platelets was negligible both in the absence and presence of bexarotene (Fig. 3) and was significantly increased by thrombin or CRP treatment. Again, the effect of CRP was significantly augmented in the presence of bexarotene (Fig. 3).

Fig. 4. Bexarotene sensitive thrombin- and CRP-induced caspase-3 activity. A-C. Original histogram overlays of fluorescence reflecting caspase-3 activity in murine platelets (A) without and (B) with a 10 min thrombin (0.01 U/ml) treatment or (C) with a 10 min CRP (5 µg/ml) treatment without (grey areas) and with (black lines) presence of bexarotene (6 µg/ml, 30 min). D. Arithmetic means $\pm$ SEM (n = 6) of the percentage of caspase-3-FITC positive murine platelets without (left bars, resting) and with a 10 min thrombin (0.01 U/ml) treatment (middle bars) or a 10 min CRP (5 µg/ml) treatment (right bars) in the absence (white bars) and presence (black bars) of 6 µg/ml bexarotene. *(p<0.05) indicates statistically significant difference from absence of bexarotene, ###(p<0.001) indicates statistically significant difference from absence of thrombin and CRP.

Fig. 5. Bexarotene sensitive thrombin- and CRP-induced cell membrane scrambling. A-C. Original histogram overlays of annexin-V-binding reflecting phosphatidylserine abundance at the surface of murine platelets (A) without and (B) with a 10 min thrombin (0.01 U/ml) treatment or (C) with a 10 min CRP (5 µg/ml) treatment without (grey areas) and with (black lines) presence of bexarotene (6 µg/ml, 30 min). D. Arithmetic means $\pm$ SEM (n = 6) of annexin-V-binding reflecting phosphatidylserine abundance at the surface of murine platelets without (left bars, resting) and with a 10 min thrombin (0.01 U/ml) treatment (middle bars) or a 10 min CRP (5 µg/ml) treatment (right bars) in the absence (white bars) and presence (black bars) of 6 µg/ml bexarotene. *(p<0.05), *** (p<0.001) indicates statistically significant difference from absence of bexarotene, ###(p<0.001) indicates statistically significant difference from absence of thrombin and CRP.

A kit has been used for the detection of activated caspase 3. As illustrated in Fig. 4, caspase activity was negligible in untreated platelets, irrespective of the presence of bexarotene. Thrombin or CRP significantly enhanced caspase activity, an effect again significantly augmented in the presence of bexarotene (Fig. 4).

Phosphatidylserine abundance was estimated from annexin-V-binding. As illustrated in Fig. 5, the percentage of annexin-V positive platelets was again negligible in untreated platelets, irrespective of the presence of bexarotene. Thrombin or CRP significantly enhanced

Fig. 6. Bexarotene sensitive thrombin- and CRP-induced cell shrinkage. A-C. Original histogram overlays of forward scatter reflecting cell volume of murine platelets (A) without and (B) with a 10 min thrombin (0.01 U/ml) treatment or (C) with a 10 min CRP (5 µg/ml) treatment without (grey areas) and with (black lines) presence of bexarotene (6 µg/ml, 30 min). D. Arithmetic means $\pm$ SEM (n = 6) of forward scatter reflecting cell volume of murine platelets without (left bars, resting) and with a 10 min thrombin (0.01 U/ml) treatment (middle bars) or a 10 min CRP (5 µg/ml) treatment (right bars) in the absence (white bars) and presence (black bars) of 6 µg/ml bexarotene. * (p<0.05), ** (p<0.01) indicates statistically significant difference from absence of bexarotene, ### (p<0.001) indicates statistically significant difference from absence of thrombin and CRP.

the percentage of annexin-V binding platelets, an effect again significantly augmented in the presence of bexarotene (Fig. 5).

Platelet volume was estimated from forward scatter, which was determined by flow cytometry. As illustrated in Fig. 6, thrombin or CRP treatment was followed by a marked decrease of forward scatter, an effect again significantly decreased in the presence of bexarotene (Fig. 6).

Discussion

The present observations demonstrate that bexarotene significantly augments the effect of thrombin on cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), annexin-V-binding, cell volume, and caspase activity as well as the effect of collagen related peptide (CRP) on $[\text{Ca}^{2+}]_i$, P-selectin abundance, activated $\alpha_{\text{IIb}}\beta_3$ integrin, annexin-V-binding, cell volume, and caspase activity. Thus, bexarotene sensitizes the platelets for the effects of thrombin and CRP. In the absence of thrombin and CRP bexarotene has only a slight, but statistically significant enhancing effect on $[\text{Ca}^{2+}]_i$.

The augmenting effects of bexarotene on platelet activation and apoptosis may, at least in part, be secondary to the augmented increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [53, 63]. An increase of $[\text{Ca}^{2+}]_i$ is followed by platelet activation and may thus foster the development of arterial thrombosis [52]. Increased $[\text{Ca}^{2+}]_i$ is further a stimulator of cell membrane scrambling with translocation of phosphatidylserine to the platelet surface [50, 64-66]. Phosphatidylserine abundance at the platelet surface fosters adhesion, coagulation and hemostasis [67]. Phosphatidylserine exposing platelets could be further bound to and engulfed by macrophages [68].

In conclusion, bexarotene augments the thrombin- and/or CRP-induced increase of $[\text{Ca}^{2+}]_i$, P-selectin abundance, $\alpha_{\text{IIb}}\beta_3$ integrin activity, annexin-V-binding, and caspase activity. Thus, bexarotene contributes to the triggering of platelet activation and apoptosis.

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Disclosure Statement

The authors of this manuscript state that they have no conflicts of interest to declare.

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