The NF κ B Pathway Inhibitors Bay 11-7082 and Parthenolide Induce Programmed Cell Death in Anucleated Erythrocytes

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Key Words

Cell volume • Eryptosis • Calcium • Phosphatidylserine exposure • Glutathione • N-acetylcysteine

Abstract

The preclinical compounds Bay 11-7082 and parthenolide trigger apoptosis, an effect contributing to their antiinflammatory action. The substances interfere with the activation and nuclear translocation of nuclear factor NF κ B, by inhibiting NF κ B directly (parthenolide) or by interfering with the inactivation of the NF κ B inhibitory protein I κ B- α (Bay 11-7082). Beyond that, the substances may be effective in part by nongenomic effects. Similar to apoptosis of nucleated cells, erythrocytes may undergo apoptosis-like cell death (eryptosis) characterized by cell membrane scrambling with phosphatidylserine exposure, and cell shrinkage. Thus, erythrocytes allow the study of nongenomic mechanisms contributing to suicidal cell death, e.g. Ca²⁺ leakage or glutathione depletion. The present study utilized Western blotting to search for NF κ B and I κ B- α expression in erythrocytes, FACS analysis to determine cytosolic Ca²⁺ (Fluo3 fluorescence), phosphatidylserine

exposure (annexin V binding), and cell volume (forward scatter), as well as an enzymatic method to determine glutathione levels. As a result, both NF κ B and I κ B- α are expressed in erythrocytes. Targeting the NF κ B pathway by Bay 11-7082 (IC₅₀ $\approx$ 10 μ M) and parthenolide (IC₅₀ $\approx$ 30 μ M) triggered suicidal erythrocyte death as shown by annexin V binding and decrease of forward scatter. Bay 11-7082 treatment further increased intracellular Ca²⁺ and led to depletion of reduced glutathione. The effects of Bay 11-7082 and parthenolide on annexin V binding could be fully reversed by the antioxidant N-acetylcysteine. In conclusion, the pharmacological inhibitors of NF κ B, Bay 11-7082 and parthenolide, interfere with the survival of erythrocytes involving mechanisms other than disruption of NF κ B-dependent gene expression.

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Introduction

Nuclear factor κ B (NF κ B) is known as a transcription factor fostering cell survival [1-3]. It is bound to the inhibitory protein I κ B, which retains NF κ B within the cytosol thus preventing its transcriptional activity. I κ B

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phosphorylation by I κ B kinase (IKK) prepares I κ B for ubiquitination and subsequent degradation. Pharmacological inhibition of IKK by Bay 11-7082 or of NF κ B by parthenolide trigger apoptosis [4-7]. Beyond that, the substances may in addition compromise cell function and survival by interference with NF κ B-independent mechanisms, such as caspase 1 [8, 9]. Most recently, expression of NF κ B was observed in blood platelets [10]. As blood platelets lack nuclei, NF κ B signaling in those cells hardly involves genomic regulation.

Human erythrocytes similarly lack nuclei and their function cannot be modified by genomic regulation. Yet, erythrocytes may undergo apoptosis-like suicidal cell death or eryptosis [11, 12]. In analogy to apoptosis of nucleated cells, eryptosis is paralleled by cell shrinkage, cell membrane blebbing and cell membrane scrambling resulting in exposure of phosphatidylserine at the erythrocyte surface [11, 13, 14]. The cell membrane scrambling could be stimulated by increase of cytosolic Ca²⁺ activity [13, 14]. Ca²⁺ may enter erythrocytes through Ca²⁺-permeable cation channels [15-17]. Beyond its effect on cell membrane scrambling, the increase of Ca²⁺ leads to activation of Ca²⁺-sensitive K⁺ channels [18, 19] with subsequent exit of KCl and osmotically obliged water and thus to erythrocyte shrinkage [20].

Eryptosis is enhanced in several clinical conditions, including iron deficiency [21], sickle-cell anemia [22, 23], beta-thalassemia [24], glucose-6-phosphate dehydrogenase (G6PD)-deficiency [24], Hemolytic Uremic Syndrome [25], sepsis [26], malaria [27-31] and Wilson's disease [32]. Moreover, eryptosis is triggered or modified by a wide variety of substances [11, 33-38].

The present study explored whether NF κ B and I κ B- α are expressed in nucleus-free red blood cells, and whether the antiinflammatory and anticancer substances Bay 11-7082 and parthenolide modify the survival of human erythrocytes. For the first aim, we used highly purified erythrocyte concentrates with defined cellular compositions, and whole blood versus its corresponding purified erythrocyte fraction, and analysed the expression of the transcription factor NF κ B and its inhibitory upstream regulator I κ B- α by Western blot analysis. Second, we investigated the functional role of these proteins in erythrocyte programmed cell death (eryptosis) by targeting the NF κ B signaling pathway with the pharmacological inhibitors Bay 11-7082 and parthenolide. Finally, we further explored the underlying mechanism of Bay 11-7082- and parthenolide-induced eryptosis, and determined the Bay 11-7082-dependent oxidative status in erythrocytes.

Materials and Methods

Human erythrocytes

Leukocyte-depleted erythrocyte concentrates provided by the blood bank of the University of Tübingen (Ottfried-Müller Str. 4/1, 72076 Tübingen, Germany) were used. The volunteers providing erythrocytes gave informed consent. The study has been approved by the Ethical commission of the University of Tübingen (343/2008BO2). Analyses of blood cell numbers (white blood cells (WBCs), red blood cells (RBCs) and platelets (PLTs)) in whole blood and in erythrocyte concentrates were performed by standard laboratory procedures by the main laboratory of the Department of Dermatology of the University Medical Center Tübingen (Tübingen, Germany).

Incubations and solutions

For the *in vitro* experiments on suicidal death of erythrocytes, incubations were carried out at 37°C in Ringer solution at a hematocrit of 0.4% in a total volume of 2 ml. Ringer solution contained (in mM): 125 NaCl, 5 KCl, 1 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH, 5 glucose, 1 CaCl₂, pH 7.4. Where indicated, Bay 11-7082 (Sigma, München, Germany) or parthenolide (Biomol, Hamburg, Germany) were added at the indicated concentrations and incubation times. Bay 11-7082, parthenolide and ionomycin (Sigma) were dissolved in final concentrations of 0.2% dimethyl sulfoxide (DMSO). N-acetylcysteine (NAC) was purchased from Sigma and dissolved in aqua bidest with a final solvent concentration of 0.2%. Solvent-treated erythrocytes served as negative controls.

Hemolysis

Erythrocyte concentrates (0.4% hematocrit) were treated with varying concentrations of Bay 11-7082 or parthenolide, and hemolysis was determined as described previously [39]. After incubation, the samples were centrifuged for 4 min at 400 x g, 4 °C, and the supernatants were harvested. To quantify hemolysis, the hemoglobin 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.

Western blotting

To examine the expression of NF κ B and I κ B- α in human erythrocytes, 250 μ l erythrocyte pellet were lysed in 50 ml of 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4). Ghost membranes were pelleted (15,000 g for 20 min at 4°C) and lysed in 250 μ l lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 1mM NaF, 1mM Na₃VO₄, 0.4% β -mercaptoethanol) containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). In all cases, cell lysates were solubilized in Laemmli sample buffer at 95°C for 5 min and resolved by 10% SDS-PAGE. For immunoblotting, proteins were electrotransferred onto a PVDF membrane and blocked with 5% nonfat milk in TBS-0.1% Tween 20 (TBST) at room temperature for 1 h. Then, the membrane was incubated with

affinity purified rabbit anti-NF κ B (1:1000; 65 kDa, Cell Signaling, Frankfurt a. M., Germany), anti-I κ B antibody (1:500; 39 kDa, Cell Signaling) or anti-P-I κ B- α (S32/36) (5A5) mouse monoclonal antibody (1:500; 39 kDa, Cell Signaling) at 4°C overnight. After washing with TBST and subsequent blocking, the blots were incubated with secondary goat-anti-rabbit/donkey-anti-mouse horse-radish-peroxidase (HRP)-conjugated antibody (1:5000; Cell Signaling) for 1 h at room temperature. After washing, antibody binding was detected with the ECL detection reagent (Amersham, Freiburg, Germany) as described [30]. Extracts from unstimulated HeLa cells were used as background controls, and extracts from TNF-treated HeLa cells served as fully stimulated positive controls for NF κ B expression.

Phosphatidylserine exposure and forward scatter

Erythrocyte concentrates (0.4 % hematocrit) were treated with varying concentrations of NF κ B pathway inhibitors Bay 11-7082 and parthenolide. After incubation, 100 μ l of the cells were washed in annexin binding buffer containing (in mM): 125 NaCl, 10 HEPES (pH 7.4) and 5 CaCl₂. Erythrocytes were then stained with Annexin-Fluos (Roche Diagnostics) at a 1:35 dilution and mixed gently on a vortex mixer. FITC-labelled annexin V binds to phosphatidylserine (PS) being exposed on the outer leaflet of the plasma membrane. After 20 min incubation in the dark at room temperature, samples were diluted 1:5, thoroughly mixed to achieve single-cell suspensions, and measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson, Heidelberg, Germany). Cells were analysed by forward scatter (FSC) and annexin-fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm as described earlier [17].

Fluorescence microscopy

Fluorescence microscopy was performed essentially as described [40]. After incubation with 10 μ M Bay 11-7082 or DMSO-containing Ringer solution (control), erythrocytes were stained with Annexin-Fluos as described above. After washing the cells with annexin buffer, 10 μ l of the suspension were applied to a slide and covered with a cover glass. Finally, the cells were analyzed under a Leica TCS-SP/Leica DM RB confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with Leica Confocal Software LCS (Version 2.61).

Measurement of intracellular Ca²⁺

Erythrocytes were loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) essentially as described earlier [26]. For this, erythrocytes (0.4 % hematocrit), suspended in 6 ml Fluo3/AM-loading buffer containing (in mM): 123 NaCl, 5 KCl, 1 MgSO₄, 25 HEPES (pH 7.4), 10 glucose, 10 sodium pyruvate, and 2 CaCl₂ were loaded by addition of 12 μ l Fluo3/AM (Calbiochem, Bad Soden, Germany) from a Fluo3/AM stock solution (2.0 mM in DMSO). The cells were incubated at 37 °C for 20 min under shaking and protection from light. An additional 12 μ l of Fluo3/AM stock solution was

added, with incubation carried out for further 40 min without shaking. Fluo3/AM-loaded erythrocytes were centrifuged at 1500 rpm for 5 min at 22 °C. Excess dye was removed by washing erythrocytes two times with Fluo3/AM-loading buffer and once with Ringer solution. For flow cytometry, Fluo3/AM-loaded erythrocytes were resuspended in Ringer solution containing Bay 11-7082 (0 - 20 μ M), parthenolide (0-100 μ M), or the Ca²⁺ ionophor ionomycin (0.1 μ M; see Fig. 4A and B) as a positive control, or vehicle alone. After incubation for different time periods at 37 °C, Ca²⁺-dependent fluorescence intensity was measured in the fluorescence channel FL-1.

Glutathione measurement

GSH refers to the reduced form of glutathione and GSSG to the oxidized disulfide dimer. GSx refers to the tripeptide unit of unspecified oxidation state (GSH equivalents) and was quantified in a microtiter plate according to the technique of Dringen and Hamprecht [41] using the method originally described by Tietze [42]. The samples (cell extracts after incubation with Bay 11-7082) and standards were processed through exactly the same procedure. The samples and standards were kept on ice until being loaded into the microtiter plate. 10 μ l of the cell lysates or standards containing 0-600 pmol of GSx/10 μ l were transferred into wells of microtiter plates and diluted with 90 μ l of water, yielding final concentrations of 0.1% sulfosalicylic acid to precipitate the proteins in the samples. After addition of 100 μ l of reaction mixture (0.1 M sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.3 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 0.4 mM NADPH, and 1 U/ml glutathione reductase), the increase in extinction at 405 nm was detected in 30 s intervals over a range of 5 min using a microtiter plate reader (Multiscan EX; Thermo Electron Corporation, Erlangen, Germany). Glutathione contents were evaluated by using a calibration curve established with standard samples. Glutathione disulfide was quantified after derivatization of reduced glutathione with 2-vinylpyridine (2VP) to conjugate GSH before assaying of residual GSSG. In brief, 130 μ l of the protein-free supernatant was mixed with 5 μ l of undiluted 97% 2VP and adjusted with 0.2 M Tris-Base (Sigma-Ultra) to pH 5. Standard samples with defined GSSG concentrations were treated the same way. After a 1 h incubation at room temperature, 10 μ l of the 2VP-treated samples or standards were assayed as described above using a calibration curve established between 0 and 600 pmol of GSSG per well. The extinction at 405 nm was followed over a 5 min period in 30 s intervals. Erythrocytes concentrates (0.4 % hematocrit) were treated with varying concentrations of Bay 11-7082. After 24 h incubation, the cells were washed two times with PBS and assayed as described above.

Statistics

Data are expressed as arithmetic means $\pm$ SEM and statistical analyses were made by ANOVA. $p < 0.05$ was considered as statistically significant.

Fig. 1. Expression of NFκB and IκB-α in erythrocytes. A. Original Western blot demonstrating the expression of NFκB (upper panels) and IκB-α protein (lower panels) in erythrocyte concentrates from 4 different individuals (p1-p4). White blood cell (WBC), red blood cell (RBC) and platelet (PLT) numbers of the different samples are given in the table on top. As indicated in the figure, 5, 10, 20 and 30 μl of erythrocyte lysates were loaded per lanes of the gels. HeLa cells treated with (+) or without (-) TNF were used as controls. Note that the percentage of contaminating cells in the erythrocyte concentrates is below 1 %. B. Original Western blot of protein extracts (30 μl per lane) demonstrating the expression of NFκB (upper panel) and IκB-α protein (middle panel) from whole blood (lane 1), diluted whole blood (1:15; lane 2), or purified erythrocytes (lane 3). Western blot of β-actin was used as a loading control. White blood cell (WBC), red blood cell (RBC) and platelet (PLT) numbers of whole blood (WB) and its corresponding purified erythrocytes (RBC) are given in the table on top. Note that the purified erythrocyte preparation contained 3.7 % platelets and 6.8 % white blood cells of the original whole blood sample. p65: 65 kDa subunit of NFκB; p39: 39 kDa IκB-α single chain; p40: 40 kDa β-actin single chain.

Fig. 2. The NFκB pathway inhibitor Bay 11-7082 induces phosphatidylserine exposure and cell shrinkage. A. Original histograms of phosphatidylserine exposure (annexin V binding) in untreated erythrocytes (left panel) and in erythrocytes treated for 48 hours with 10 μM Bay 11-7082 (right panel). B. Arithmetic means $\pm$ SEM (n=12) of the percentage of annexin V binding erythrocytes after 24 hours (closed squares) or 48 hours (open squares) incubation as a function of Bay 11-7082 concentration. #indicates significant difference from the respective values in the absence of the drug. C. Arithmetic means $\pm$ SEM (n=12) of the erythrocyte forward scatter in % of control after a 24 hours (closed squares) or 48 hours (open squares) incubation as a function of Bay 11-7082 concentration. #indicates significant difference from the respective values in the absence of the drug. D. Transmission light microscopy (upper panels) and annexin V fluorescence (lower panels) of erythrocytes incubated for 48 hours without (left panels) or with (right panels) 10 μM Bay 11-7082. The insert depicts an erythrocyte at higher magnification. Bars: 50 μm. Insert bars: 10 μm. E. Arithmetic means $\pm$ SEM (n=12) of hemolysis (in % of maximal hemolysis after exposure to distilled water) from erythrocytes following a 24 hours (closed squares) or a 48 hours (open squares) incubation as a function of Bay 11-7082 concentration. #indicates significant difference from the respective values in the absence of the drug.

Results

According to Western blotting, highly purified human erythrocyte concentrates from 4 different volunteers expressed both, NFκB and IκB-α protein (Fig. 1A). The

erythrocyte concentrates were virtually free of white blood cells (WBCs in Fig. 1A, Upper Table) and contained less than 1 % platelets (PLTs in Fig. 1A, Upper Table). This makes it unlikely that the observed NFκB and IκB-α protein expression originates from WBC or

PLT contaminations. To further exclude that the immuno-positive protein bands originate from other cells than erythrocytes, we performed a purification and dilution experiment. For this, cell preparations were obtained from whole blood (WB) and from corresponding erythrocytes (RBC) which have been purified by centrifugation, and the numbers of the different cell types in both preparations were determined (Fig. 1B, Upper Table). Since the purified erythrocyte preparation contained 3.7 % platelets and 6.8 % white blood cells of the original whole blood sample, the whole blood extract was additionally diluted by 1:15 and loaded on the gel. As shown in Fig. 1B, the diluted extract from whole blood (lane 2) did not give rise to an immuno-reactive band although it contained at least as much white blood cells and platelets as the undiluted purified RBC preparation (lane 3). Thus, the immuno-reactive band in the purified RBC preparation (lane 3) could not originate from contaminating WBCs or PLTs.

Additional experiments explored the effects on erythrocytes of two pharmacological substances known to interfere with NF κ B signaling. As illustrated in Fig. 2A and B, exposure of erythrocytes to Bay 11-7082 was followed by a marked increase of annexin V binding erythrocytes, a finding pointing to stimulation of cell membrane scrambling. A dose response curve of the annexin V binding yielded a halfmaximal effect at 11 μ M Bay 11-7082 (Fig. 2B) after 48 h treatment. Furthermore, the forward scatter was determined as an indicator of cell volume changes. The exposure to Bay 11-7082 was indeed followed by a significant decrease of forward scatter, an observation pointing to cell shrinkage (Fig. 2C). To possibly disclose Bay 11-7082-induced hemolysis, drug- and vehicle-treated cells were stained with FITC-labelled annexin V and then analyzed by fluorescence microscopy. Whereas control erythrocytes showed a normal disc-like shape with very low numbers of cells being annexin V-positive (Fig. 2D, upper left and lower left panel), the majority of Bay 11-7082-treated erythrocytes were annexin V-positive and displayed a shrunken phenotype (Fig. 2D, upper right and lower right panel). The cell membrane of those cells remained intact and annexin V binding showed a pattern of raft-like structures (Fig. 2D, upper right and upper left insert). To additionally quantify hemolysis, the hemoglobin concentration was determined in the supernatant of erythrocytes following exposure to different concentrations of Bay 11-7082. As shown in Fig. 2E, Bay 11-7082 at concentrations > 7.5 μ M showed a significant induction of hemolysis after 24 h which further increased after 48 h. However, hemolysis was approx. 8 % of the total population, and thus ten-fold lower as

Fig. 3. The NF κ B inhibitor parthenolide induces phosphatidylserine exposure and cell shrinkage. A. Original histograms of phosphatidylserine exposure (annexin V binding) in untreated erythrocytes (left panel) and in erythrocytes treated for 48 hours with 30 μ M parthenolide (right panel). B. Arithmetic means $\pm$ SEM (n=12) of the percentage of annexin V binding erythrocytes after 24 hours (closed squares) or 48 hours (open squares) incubation as a function of parthenolide concentration. #indicates significant difference from the respective values in the absence of the drug. C. Arithmetic means $\pm$ SEM (n=12) of the erythrocyte forward scatter in % of control after a 24 hours (closed squares) or 48 hours (open squares) incubation as a function of parthenolide concentration. #indicates significant difference from the respective values in the absence of the drug. D. Arithmetic means $\pm$ SEM (n=12) of hemolysis (in % of maximal hemolysis after exposure to distilled water) from erythrocytes following a 24 hours (closed squares) or a 48 hours (open squares) incubation as a function of parthenolide concentration. #indicates significant difference from the respective values in the absence of the drug.

compared with the effect of Bay 11-7082 on annexin V-binding (compare Fig. 2B and 2E).

Similar to Bay 11-7082, parthenolide triggered annexin V binding of erythrocytes (Fig. 3A, B), indicating that parthenolide likewise stimulated cell membrane scrambling with a halfmaximal effect at 30 μ M

Fig. 4. Effect of Bay 11-7082 and parthenolide on cytosolic Ca^{2+} . **A.** Original histograms of Fluo3-dependent fluorescence in DMSO-treated erythrocytes (left, control) and in erythrocytes treated for 6 hours with 10 μM Bay 11-7082 (middle panel) or with 0.1 μM ionomycin (right panel) as a positive control. Numbers depict the geometric mean (GeoMean) of the Fluo3-dependent fluorescence of the different erythrocyte populations. **B.** Upper panel: Arithmetic means $\pm$ SEM ($n = 9$) of Fluo3 fluorescence in erythrocytes following exposure for 1 (open squares), 3 (open triangles) and 6 hours (closed squares) to Ringer solution containing increasing concentrations of Bay 11-7082. # indicates significant difference from the respective values in the absence of the drug. Maximum Fluo3-dependent fluorescence of ionomycin-treated erythrocytes is shown on the right. Lower panel: Dose-response curve of the concomitant effect of Bay 11-7082 on forward scatter (FSC) of the erythrocyte populations. Arithmetic means $\pm$ SEM ($n = 9$) of the FSC GeoMean following 6 hours of treatment with different concentrations of Bay 11-7082. # indicates significant difference from the respective values in the absence of the drug. The decrease of FSC of ionomycin-treated erythrocytes is shown on the right. **C.** Arithmetic means $\pm$ SEM ($n = 8$) of Fluo3 fluorescence in erythrocytes following exposure for 6 hours (closed squares) to Ringer solution containing increasing concentrations of parthenolide. # indicates significant difference from the respective values in the absence of the drug. Maximum Fluo3-dependent fluorescence of ionomycin-treated erythrocytes in these experiments is shown on the right.

parthenolide (Fig. 3B) after 48 h treatment. Challenge of erythrocytes with parthenolide was also followed by a significant decrease of the forward scatter, an effect reflecting cell shrinkage (Fig. 3C). As illustrated in Fig. 3D, parthenolide also induced significant hemolysis at the concentrations used. However, as in the case of Bay 11-7082, this effect of parthenolide was small as compared with its corresponding influence on phosphatidylserine exposure (compare Fig. 3B and 3D).

In a next series of experiments, Fluo3 fluorescence has been employed to estimate the cytosolic Ca^{2+} concentration. As illustrated in Fig. 4A and B, the exposure of erythrocytes to Bay 11-7082 was followed by a significant increase of Fluo3 fluorescence pointing to an increase of cytosolic Ca^{2+} . Fig. 4B displays a dose response curve of the Bay 11-7082-induced effect. The concentration to yield halfmaximum effects on the cytosolic Ca^{2+} level after 6 h of drug treatment was below 10 μM Bay 11-7082. At the highest concentration of Bay 11-7082 (20 μM), intracellular Ca^{2+} was approx. a third of the maximum calcium load induced by the Ca^{2+} ionophore ionomycin (Fig. 4A, B). An increase of cytosolic Ca^{2+} is expected to shrink erythrocytes due to activation of Ca^{2+} -

Fig. 5. Bay 11-7082 does not affect $\text{I}\kappa\text{B-}\alpha$ phosphorylation. **A.** Original Western blot demonstrating the expression of the phosphorylated form of $\text{I}\kappa\text{B-}\alpha$ (P-I $\kappa\text{B-}\alpha$) protein (upper panel) in erythrocyte concentrates treated for 6 h with DMSO (left) or 20 μM Bay 11-7082 (right). Actin (lower panel) was used as loading control. **B.** Quantification of the bands by densitometry. The P-I $\kappa\text{B-}\alpha$ /actin ratio of DMSO-treated erythrocytes was set as 1.

Fig. 6. Bay 11-7082 depletes reduced glutathione (GSH). Dose-response curve of the effect of Bay 11-7082 on reduced glutathione (GSH) level. Arithmetic means $\pm$ SEM ($n = 3-6$) of the effect of Bay 11-7082 on GSH level of erythrocytes exposed for 24 hours to Ringer with Bay 11-7082 as a function of drug concentration. # indicates significant difference from the respective values in the absence of the drug.

sensitive K^+ channels [11, 20]. Thus, in these experiments, the forward scatter was determined in parallel as an indicator of cell volume changes. Indeed, the exposure of erythrocytes to Bay 11-7082 for 6 h was followed by a decrease of the forward scatter (Fig. 4B, lower panel). Similar to Bay 11-7082, parthenolide concentration-dependently increased the Ca^{2+} load of erythrocytes after 6 h (Fig. 4C), indicating that parthenolide induces phosphatidylserine exposure by a Ca^{2+} -mediated pathway.

Additional experiments aimed to gain some insight into the mechanism underlying Bay 11-7082-induced eryptosis. Based on the experiments demonstrating a maximum increase of intracellular Ca^{2+} levels, erythrocytes were treated with 20 μ M Bay 11-7082 for 6 h. Then, the phosphorylation status of I κ B- α in vehicle- and Bay 11-7082-treated erythrocytes was determined by Western blot and analyzed via densitometry. However, Bay 11-7082 did not affect the phosphorylation status at Ser32/36 of I κ B- α under these experimental conditions as compared with vehicle-treated controls (Fig. 5). As an alternative mechanism, we hypothesized that the effect of Bay 11-7082 involved oxidative stress, which is known to trigger eryptosis at least in part by activation of Ca^{2+} -permeable cation channels [11]. To test this hypothesis, we determined the level of reduced glutathione (GSH) after treatment with different concentrations of Bay 11-7082. As shown in Fig. 6, Bay 11-7082 at concentrations above 7.5 μ M fully depleted GSH.

Fig. 7. Antagonism of Bay 11-7082- and parthenolide-induced annexin V binding and cell shrinkage by N-acetylcysteine (NAC). A. Histogram of annexin V binding in a representative experiment of erythrocytes exposed for 24 hours to 10 μ M Bay 11-7082 in the absence (left) or presence (right) of 1 mM N-acetyl-cysteine (NAC). B. Arithmetic means $\pm$ SEM ($n = 12$) of the percentage of annexin V-binding erythrocytes following exposure for 24 hours to Ringer supplemented with different concentrations of Bay 11-7082 (0 – 20 μ M) in the presence (closed squares) and absence (open squares) of 1 mM NAC. Annexin V-binding in control cultures was 1.56 ± 0.06 % in the absence of NAC, and 1.47 ± 0.07 % in the presence of 1 mM NAC. * indicates significant difference from the respective values in the absence of the NAC. C. Arithmetic means $\pm$ SEM ($n = 12$) of the forward scatter (FSC in % of control) of erythrocytes treated for 24 hours with different concentrations of Bay 11-7082 (0 – 20 μ M) in the presence (closed squares) and absence (open squares) of 1 mM NAC. The forward scatter (arbitrary units) in control cultures was 420 ± 8 in the absence of NAC, and 409 ± 7 in the presence of 1 mM NAC. * indicates significant difference from the respective values in the absence of the NAC. D. Arithmetic means $\pm$ SEM ($n = 9$) of the percentage of annexin V-binding erythrocytes following exposure for 48 hours to Ringer supplemented with different concentrations of parthenolide (0 – 100 μ M) in the presence (closed squares) and absence (open squares) of 1 mM NAC. Annexin V-binding in control cultures was 3.12 ± 0.71 % in the absence of NAC, and 4.45 ± 0.93 % in the presence of 1 mM NAC. * indicates significant difference from the respective values in the absence of NAC.

If Bay 11-7082 and parthenolide would be effective by indirectly inducing oxidative stress [43], the applica-

tion of a reducing substance should blunt Bay 11-7082- or parthenolide-induced eryptosis. Thus, experiments were performed in the absence and presence of the reducing substance N-acetylcysteine (NAC). As illustrated in Fig. 7A,B and Fig. 7D, the addition of 1 mM NAC virtually abolished the effect of 5 - 20 μ M Bay 11-7082 and 20 - 100 μ M parthenolide on annexin V binding. Similarly, NAC pretreatment prevented Bay 11-7082-induced cell shrinkage as evidenced by normalization of the forward scatter (Fig. 7C). Accordingly, prevention of oxidative stress suppressed the eryptotic effect of Bay 11-7082 and parthenolide.

Discussion

The present observations reveal completely novel stimulators of suicidal erythrocyte death. They demonstrate that the antiinflammatory and anticancer substances Bay 11-7082 and parthenolide trigger cell membrane scrambling and cell shrinkage, the two hallmarks of eryptosis [11]. They further reveal that the effect is paralleled by increase of cytosolic Ca^{2+} and reversed by the reducing substance N-acetylcysteine. The Ca^{2+} -permeable cation channels could be activated by oxidative stress [11, 17] and the subsequent entry of Ca^{2+} could contribute to the stimulation of cell membrane scrambling and triggering of cell shrinkage [11].

It is tempting to speculate that the observed effects of Bay 11-7082 and parthenolide are related to their interference with NF κ B signaling. NF κ B is known to confer survival of nucleated cells, an effect attributed to the effect of NF κ B on transcription [1-3]. NF κ B is bound to the inhibitory protein I κ B, which retains NF κ B within the cytosol thus preventing its transcriptional activity. Bay 11-7082 inhibits IKK and parthenolide inhibits NF κ B, effects considered to contribute to their negative effect on cell survival [7]. Considering the restricted specificity of Bay 11-7082 and parthenolide [8, 9, 44-46], and the lack of an inhibitory effect of Bay 11-7082 on the phosphorylation status of I κ B- α , the pharmacological evidence in favour of a functional role for NF κ B in the regulation of erythrocyte survival is limited. Nevertheless, the present study reveals that erythrocytes do harbour the transcription factor NF κ B and I κ B- α even though they lost their nuclei, and the theoretical possibility should be considered that NF κ B and I κ B- α serve some function other than regulation of transcription.

Bay 11-7082 and parthenolide may in part be effective through mechanisms other than regulation of NF κ B, which may be relevant for survival of both erythrocytes and nucleated cells. The present observations point to a role of oxidative stress, which is known to challenge the survival of erythrocytes [47, 48]. Oxidative stress activates the Ca^{2+} -permeable cation channels, thus triggering Ca^{2+} entry and Ca^{2+} -dependent stimulation of cell membrane scrambling as well as cell shrinkage [11]. In addition to cation channels, oxidative stress activates erythrocyte anion channels [49, 50]. Oxidative stress is further known to activate aspartyl and cysteinyl proteases [51], which are expressed in erythrocytes [52], to cleave the anion exchanger band 3 *in vitro* [52], and to stimulate cell membrane scrambling and phosphatidylserine exposure in erythrocytes [53]. Eryptosis following ionomycin or hyperosmotic shock does, however, not require the activation of caspases [13, 40].

Whatever the underlying mechanisms, accelerated eryptosis limits the life span of circulating erythrocytes. Phosphatidylserine-exposing cells are bound to [54] and engulfed by [55] macrophages. Thus, they are rapidly cleared from circulating blood [21]. Accordingly, accelerated eryptosis may lead to anemia [11].

Eryptosis may further influence microcirculation. Erythrocytes exposing phosphatidylserine at their surface bind to the vascular wall [23, 56-59] and stimulate the assembly of prothrombinase and tenase, the generation of thrombin and thus blood clotting [56, 60, 61].

In conclusion, erythrocytes express NF κ B and I κ B- α and are driven into suicidal death by Bay 11-7082 and parthenolide, both preclinical compounds interfering with NF κ B-dependent signaling. The observations reveal a novel effect of Bay 11-7082 and parthenolide, which may similarly participate in their stimulating effect on suicidal death of nucleated cells.

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