Original Paper

Stimulating Effect of Manumycin A on Suicidal Erythrocyte Death

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Key Words

Phosphatidylserine • Cell volume • Eryptosis • SB203580 • Staurosporine • Calcium • Manumycin A

Abstract

Background/Aims: The streptomycete derived farnesyltransferase inhibitor Manumycin A triggers apoptosis of tumor cells and is thus considered for the treatment of malignancy. The present study explored whether Manumycin A could similarly stimulate eryptosis, the suicidal death of erythrocytes characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Stimulators of eryptosis include Ca^{2+} entry as well as activation of staurosporine sensitive protein kinase C and SB203580 sensitive p38 kinase. The present study explored, whether Manumycin A induces eryptosis and, if so, to shed some light on the mechanisms involved. **Methods:** Phosphatidylserine abundance at the human erythrocyte surface was estimated from annexin-V-binding, cell volume from forward scatter, and hemolysis from hemoglobin concentration in the supernatant. **Results:** A 48 hours exposure of human erythrocytes to Manumycin A ($\geq 5 \mu\text{g/ml}$) significantly increased the percentage of annexin-V-binding cells, significantly decreased forward scatter and significantly increased hemolysis. The effect of Manumycin A on annexin-V-binding was significantly blunted by removal of extracellular Ca^{2+} , by addition of staurosporine ($1 \mu\text{M}$) and by addition of SB203580 ($2 \mu\text{M}$). **Conclusions:** Manumycin A triggers hemolysis, cell shrinkage and phospholipid scrambling of the human erythrocyte cell membrane. The effect on cell membrane scrambling was in part but not fully dependent on entry of extracellular Ca^{2+} , as well as activity of staurosporine and SB203580 sensitive kinases.

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Introduction

The natural antibiotic [1-5] Manumycin A is a streptomycete derived farnesyltransferase inhibitor [6-15], Manumycin A downregulates the release of proinflammatory cytokines and thus counteracts inflammation [7] as well as atherosclerosis [3]. Moreover, manumycin A corrects aberrant splicing of the chloride channel *Clcn1* and may thus be effective in the

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treatment of myotonic dystrophy type 1 [16]. Manumycin A triggers suicidal death of tumor cells [9, 10, 12, 17-26] and the substance is effective against diverse malignancies [6, 8, 17-22, 27-35]. Mechanisms involved in Manumycin A induced apoptosis include inhibition of Ras [5, 6, 8, 11, 13-15, 26, 27, 29, 33], HIF-1 α [8], and IkappaB kinase (IKK) [24, 32], activation of the mitochondrial apoptotic pathway [17, 21, 23], oxidative stress [18, 20, 22, 30, 31], as well as activation of caspases [9, 19, 23].

Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal death of erythrocytes characterized by cell shrinkage [36] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [37]. Triggers of eryptosis include Ca²⁺ entry with increase of cytosolic Ca²⁺ activity ([Ca²⁺]_i) [37], as well as activation of protein kinase C or of p38 kinase [37]. Eryptosis is elicited by a wide variety of xenobiotics [37-68].

The present study explored, whether exposure of human erythrocytes to Manumycin A stimulates eryptosis. To this end, erythrocytes isolated from healthy volunteers were treated with Manumycin A and cell volume, phosphatidylserine surface abundance, as well as hemolysis quantified.

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 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, 1 CaCl₂, at 37°C for 48 hours. Where indicated, erythrocytes were exposed for 48 hours to Manumycin A (Enzo Life Sciences, Lörrach, Germany). To test for an involvement of p38 kinase, erythrocytes were exposed for 48 hours to a combination of Manumycin A and p38 kinase inhibitor SB 203580 (Tocris bioscience, Bristol, UK). To test for an involvement of protein kinase C, erythrocytes were exposed for 48 hours to a combination of Manumycin A and staurosporine (Sigma Aldrich, Hamburg, Germany).

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 100 μ l cell suspension was washed in Ringer solution containing 5 mM CaCl₂ 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. 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 Manumycin A 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 Ca²⁺

After incubation, erythrocytes were washed in Ringer solution and loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl₂ and 5 μ M Fluo-3/AM. The cells were incubated at 37°C for 30 min. Ca²⁺-dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

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 Manumycin A stimulates eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and by phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface. To this end, the erythrocytes were incubated for 48 hours in Ringer solution without or with Manumycin A (1 – 10 $\mu\text{g/ml}$).

Erythrocyte volume was estimated from forward scatter which was determined utilizing flow cytometry. As illustrated in Fig. 1, Manumycin A decreased erythrocyte forward scatter, an effect reaching statistical significance at 5 $\mu\text{g/ml}$ Manumycin A concentration. Along those lines treatment of erythrocytes with Manumycin A was followed by a significant increase of the percentage of shrunken erythrocytes (Fig. 1C), an effect reaching statistical significance at 5 $\mu\text{g/ml}$ Manumycin A concentration. Manumycin A treatment simultaneously increased the percentage of swollen erythrocytes, an effect reaching statistical significance at 10 $\mu\text{g/ml}$ Manumycin A concentration (Fig. 1D).

The percentage of hemolytic erythrocytes was estimated from the hemoglobin concentration in the supernatant. As illustrated in Fig. 2, a 48 hours exposure to Manumycin A

Fig. 1. Effect of Manumycin A on erythrocyte forward scatter. A. Original histogram of erythrocyte forward scatter following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 10 $\mu\text{g/ml}$ Manumycin A. B. Arithmetic means $\pm$ SEM ($n = 16$) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Manumycin A (1 - 10 $\mu\text{g/ml}$). C. Arithmetic means $\pm$ SEM ($n = 16$) of the percentage erythrocytes with forward scatter (FSC) <200 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Manumycin A (1 - 10 $\mu\text{g/ml}$). D. Arithmetic means $\pm$ SEM ($n = 16$) of the percentage erythrocytes with forward scatter (FSC) >800 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Manumycin A (1 - 10 $\mu\text{g/ml}$). *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$) indicates significant difference from the absence of Manumycin A (ANOVA).

Fig. 2. Effect of Manumycin A on hemolysis. Arithmetic means $\pm$ SEM ($n = 8$) of the percentage hemolytic erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Manumycin A (1 - 10 $\mu\text{g/ml}$). ***($p < 0.001$) indicates significant difference from the absence of Manumycin A (ANOVA).

Fig. 3. Effect of Manumycin A 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 10 $\mu\text{g/ml}$ Manumycin A. B. Arithmetic means $\pm$ SEM ($n = 16$) of the percentage annexin-V-binding erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Manumycin A (1 - 10 $\mu\text{g/ml}$). **($p < 0.01$), ***($p < 0.001$) indicates significant difference from the absence of Manumycin A (ANOVA).

increased the percentage of hemolytic erythrocytes, an effect reaching statistical significance at 5 $\mu\text{g/ml}$ Manumycin A.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. As illustrated in Fig. 3, a 48 hours exposure to Manumycin A increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 5 $\mu\text{g/ml}$ Manumycin A.

Fluo3 fluorescence was employed in an attempt to measure cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). However, addition of Manumycin A increased the fluorescence even without staining of the erythrocytes with Fluo3. The interference precluded safe estimates of $[\text{Ca}^{2+}]_i$.

In order to test whether Manumycin A-induced translocation of phosphatidylserine was sensitive to extracellular Ca^{2+} , erythrocytes were incubated for 48 hours in the absence or presence of 10 $\mu\text{g/ml}$ Manumycin A in the presence or nominal absence of extracellular Ca^{2+} . As shown in Fig. 4, removal of extracellular Ca^{2+} significantly blunted the effect of Manumycin A on the percentage of annexin-V-binding erythrocytes. However, even in the absence of extracellular Ca^{2+} , Manumycin A significantly increased the percentage of annexin-V-binding erythrocytes. Thus, Manumycin A-induced cell membrane scrambling was in part, but not fully dependent on entry of extracellular Ca^{2+} .

To explore, whether the effects of Manumycin A involved protein kinase C and/or p38 kinase activity, the influence of Manumycin A on annexin-V-binding was tested in the absence or presence of protein kinase C inhibitor staurosporine (1 μM) or p38 kinase inhibitor SB 203580 (2 μM). As illustrated in Fig. 5, both staurosporine and SB203580 significantly

Fig. 4. Ca²⁺ sensitivity of Manumycin A-induced phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) Manumycin A (10 µg/ml) in the presence (A) and absence (B) of extracellular Ca²⁺. C. Arithmetic means ± SEM (n = 12) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) Manumycin A (10 µg/ml) in the presence (left bars, +Ca²⁺) and absence (right bars, -Ca²⁺) of Ca²⁺. *** (p<0.001) indicates significant difference from the absence of Manumycin A, # (p<0.05) indicates significant difference from the presence of Ca²⁺ (ANOVA).

Fig. 5. Staurosporine and SB203580 sensitivity of Manumycin A-induced phosphatidylserine exposure. A,B,C. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) Manumycin A (10 µg/ml) in the absence of kinase inhibitors (A) and in the presence of 1 µM staurosporine (B) or of 2 µM SB203580 (C). D. Arithmetic means ± SEM (n = 8) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) Manumycin A (10 µg/ml) in the absence of kinase inhibitors (left bars, control) and in the presence of 1 µM staurosporine (middle bars, StSp) or of 2 µM SB203580 (right bars, SB). *** (p<0.001) indicates significant difference from the absence of Manumycin A, ### (p<0.001) indicates significant difference from the absence of kinase inhibitors (ANOVA).

blunted the effect of Manumycin A on the percentage of annexin-V-binding erythrocytes. However, even in the presence of the inhibitors, Manumycin A significantly increased the percentage of annexin-V-binding erythrocytes. Thus, Manumycin A-induced cell membrane scrambling was in part, but not fully dependent on kinase activity.

Discussion

The present observations uncover a novel effect of Manumycin A, i.e. the triggering of eryptosis, the suicidal erythrocyte death. Exposure of erythrocytes from healthy individuals with Manumycin A was followed by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentrations required for those effects were in the range of plasma concentrations determined in Manumycin A treated mice [69] and may thus be clinically relevant. Erythrocytes may be particularly sensitive to the proeryptotic effect of Manumycin A in clinical conditions with accelerated eryptosis, such as dehydration [70], hyperphosphatemia [71], chronic kidney disease (CKD) [72-75], hemolytic-uremic syndrome [76], diabetes [77], hepatic failure [78], malignancy [37], sepsis [79], sickle-cell disease [37], beta-thalassemia [37], Hb-C and G6PD-deficiency [37], as well as Wilsons disease [80].

Interference of Manumycin A with Fluo3 fluorescence measurements precluded safe estimates of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). However, the effect of Manumycin A on cell membrane scrambling was blunted by removal of Ca^{2+} from extracellular space, indicating that the effect was partially dependent on entry of extracellular Ca^{2+} . The effect of Manumycin A on cell shrinkage may similarly be due to Ca^{2+} entry from the extracellular space leading to increase of $[\text{Ca}^{2+}]_i$ with subsequent activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [36]. It should be pointed out, however, that only a portion of treated erythrocytes underwent marked cell shrinkage. Another portion of erythrocytes even increased cell volume and some erythrocytes underwent hemolysis. The mechanism accounting for the cell swelling remained elusive. Feasible causes include ATP depletion with impairment of Na^+/K^+ ATPase leading to cellular Na^+ accumulation, cellular loss of K^+ , depolarization and entry of Cl^- .

As Manumycin A triggered cell membrane scrambling even in the absence of extracellular Ca^{2+} , the drug was effective in part by mechanisms other than Ca^{2+} entry. Additional mechanisms apparently effective were activation of staurosporine and SB203580 sensitive kinases which have previously been shown to trigger eryptosis [37].

Eryptotic erythrocytes are rapidly cleared from circulating blood [37]. Thus eryptosis of defective erythrocytes may precede and thus prevent hemolysis with release of hemoglobin, which passes the renal glomerular filter, precipitates in the acidic lumen of renal tubules, occludes nephrons and thus may lead to renal failure [81]. Eryptosis is triggered by infection with the malaria pathogen *Plasmodium* and serves to remove infected erythrocytes from circulation [37].

The removal of phosphatidylserine exposing erythrocytes from circulating blood results in anemia as soon as the loss of erythrocytes exceeds the formation of new erythrocytes by erythropoiesis [37]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [82], stimulate blood clotting and foster thrombosis [83-85]. Triggering of eryptosis may thus compromise microcirculation [83, 86-90].

In conclusion, Manumycin A triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect in part dependent on Ca^{2+} entry and activation of staurosporine and SB203580 sensitive kinases.

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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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