

Mechanisms of xenobiotic-sensitive apoptotic cell death of erythrocytes

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Contents

1. ABBREVIATIONS	07
2. SUMMARY	10
3. ZUSAMMENFASSUNG	12
4. INTRODUCTION	15
4.1. Erythrocytes	15
4.2. Erythropoiesis	16
4.3. Anemia	16
4.4. Erythrocyte membrane composition	17
4.5. Ionic transportation in Erythrocyte	18
4.6. Apoptosis	20
4.7. Eryptosis	22
4.7.1. Signaling pathways of eryptosis	24
4.7.2. Eryptosis inducers	27
4.7.3. Diseases associated with enhanced eryptosis	27
4.7.4. Inhibitors of eryptosis	27
4.7.5. Consequences related to eryptosis	28
4.8. Xenobiotics	28
4.9. Mycotoxins	29

4.9.1. Enniatin A-----	30
4.9.2. Ochratoxin A-----	32
4.9.3. Zearalenone-----	34
4.10. Phytochemicals-----	36
4.10.1. Withaferin A-----	36
4.10.2. Oridonin-----	38
4.10.3. Dicoumarol-----	40
5. AIM OF THE STUDY-----	43
6. MATERIAL AND METHODS-----	42
6.1. Stimulation of suicidal death of erythrocytes by mycotoxins-----	44
6.1.1. Erythrocytes, solutions and chemicals-----	44
6.1.2. FACS analysis of annexin V-binding and forward scatter-----	44
6.1.3. Measurement of intracellular Ca ²⁺ -----	45
6.1.4. Determination of ceramide formation-----	45
6.1.5. Determination of intracellular ATP concentration-----	45
6.1.6. Measurement of hemolysis-----	46
6.1.7. Cell culture and in vitro adhesion studies-----	46
6.1.8. Confocal microscopy and immunofluorescence-----	46
6.1.9. Statistics-----	46
6.2. Stimulation of suicidal death of erythrocytes by phytochemicals-----	47
6.2.1. Erythrocytes, solutions and chemicals-----	47

6.2.2. FACS analysis of annexin-V-binding and forward scatter-----	47
6.2.3. Measurement of intracellular Ca ²⁺ -----	48
6.2.4. Determination of ceramide formation-----	48
6.2.5. Determination of intracellular ATP concentration-----	48
6.2.6. Measurement of hemolysis-----	49
6.2.7. Estimation of ROS formation-----	49
6.2.8. Patch clamp recordings-----	49
6.4.9. Confocal microscopy and immunofluorescence-----	50
6.2.10. Statistics-----	50
7. RESULTS-----	51
7.1. Stimulation of suicidal death of erythrocytes by mycotoxins-----	51
7.1.1. Enniatin A-----	51
7.1.2. Ochratoxin A-----	58
7.1.3. Zearalenone-----	64
7.2. Stimulation of suicidal death of erythrocytes by phytochemicals-----	68
7.2.1. Withaferin A-----	68
7.2.2. Oridonin-----	74
7.2.3. Dicoumarol-----	80
8. DISCUSSION-----	86
8.1. Stimulation of suicidal death of erythrocytes by mycotoxins-----	86
8.1.1. Enniatin A-----	86

8.1.2. Ochratoxin A-----	88
8.1.3. Zearalenone-----	90
8.2. Stimulation of suicidal death of erythrocytes by phytochemicals-----	91
8.2.1. Withaferin A-----	92
8.2.2. Oridonin-----	93
8.2.3. Dicoumarol-----	95
REFERENCES-----	98
ACKNOWLEDGEMENTS-----	126
CURRICULUM VITAE-----	127
LIST OF PUBLICATIONS-----	128

1. ABBREVIATIONS

ACAT	:	Acyl coenzyme A Cholesterol Acyltransferase
AE1	:	Anion Exchanger 1
AIF	:	Apoptosis-inducing factor
Akt	:	Protein Kinase B
AMPA	:	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
AMPK	:	Adenosine monophosphate-activated protein kinase
ANOVA	:	Analysis of variance
APC	:	Adenomatous Polyposis Coli
ATP	:	Adenosine triphosphate
BSA	:	Bovine serum albumin
Ca ²⁺	:	Calcium
Cl ⁻	:	Chloride
CNQX	:	6-cyano-7-nitroquinoxaline-2, 3-Dione
CO ₂	:	Carbon dioxide
COX	:	Cyclooxygenase
CTL	:	Cytotoxic T lymphocytes
DCFDA	:	2',7' dichlorodihydrofluorescein diacetate
dl	:	Deciliter
DMSO	:	Dimethylsulfoxide
DNA	:	Deoxyribonucleic acid
DNQX	:	6,7-dinitro-quinoxaline-2,3-dione
EDTA	:	Ethylenediaminetetraacetic acid
EGTA	:	glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
EIPA	:	Ethylisopropylamiloride
EPO	:	Erythropoietin
ERK	:	Extracellular regulated protein kinase
FACS	:	Fluorescence-activated cell sorting
FITC	:	Fluorescein isothiocyanate
FL	:	Fluorescence channel

fL	:	Femtolitter
FSC	:	Forward scatter
G	:	Gram
Geo mean	:	Geometric mean
GLUT	:	Glucose transporter
G6PD	:	Glucose-6-Phosphat-Dehydrogenas
H	:	Hour
Hb	:	Hemoglobin
HClO ₄	:	Perchloric acid
HEPES	:	32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HUVEC	:	Human umbilical vein endothelial cells
ICAM-4	:	Intercellular Adhesion Molecule-4
IgG	:	Immunoglobulin G
IgM	:	Immunoglobulin M
JAK	:	Janus kinase
JNK	:	Jun N-terminale Kinase
K ⁺	:	Potassium
Kg	:	Kilogram
KHCO ₃	:	Potassium bicarbonate
L	:	Liter
MCV	:	Mean cell volume
MRP4	:	Multi drug resistance protein 4
mM	:	Millimolar
Na ⁺	:	Sodium
NBQX	:	1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7 - sulfonamide
NF	:	Nuclear factor
NHE	:	Na ⁺ /H ⁺ exchanger
NPPB	:	Natriuretic peptide B

NQO1	:	NAD(P)H dehydrogenase, quinone 1
O ₂	:	Oxygen
nM	:	Nanomolar
NMDG	:	N-methyl-D-glucamine
NO	:	Nitric oxide
OTA	:	Ochratoxin A
PAF	:	Platelet activating factor
PBS	:	Phosphate-buffered saline
PDK1	:	Pyruvate dehydrogenase kinase 1
PGE2	:	Prostaglandin E2
PKA	:	Protein kinase A
PLA	:	Phospholipase A
PNQX	:	1,4,7,8,9,10-hexahydro-9-methyl- 6 nitro-pyrido[3,4-f]-quinoxaline-2,3-Dione
PS	:	Phosphatidylserine
RBC	:	Red blood cell
RR	:	<i>Rabdusia rubescens</i>
SEM	:	Standard error of mean
SM	:	Sphingomyelin
tBOOH	:	t-butyl hydroperoxide
TRPC6	:	Transient receptor potential cation channel 6
ZEA	:	Zearalenone
zVAD	:	Pancaspase inhibitor
µg	:	Microgram
µL	:	Microliter
µM	:	Micromole
µm	:	Micron

2. SUMMARY

Erythrocytes are similar to nucleated cells in that they undergo suicidal death or eryptosis. Mechanism involved in eryptosis may depend on the activation of Ca^{2+} -sensitive cation channels leading to increase intracellular Ca^{2+} activity. Enhanced cytosolic Ca^{2+} concentration stimulates phosphatidylserine exposure at the cell surface and cell shrinkage. Eryptosis is a physiological process which may have an important contribution in the limitation of erythrocyte survival. Excessive eryptosis due to stress, toxicity, diseases or defective compensatory mechanism may lead to anemia. The present observations determined the role of xenobiotics in the regulation of eryptosis as well as their toxic effects for erythrocytes.

The first part of the study explored the mechanisms adopted by different foods born mycotoxins (enniatin A, ochratoxin A and zearalenone) in the triggering of eryptosis. Exposure of erythrocytes for 48 hours to enniatin A ($\geq 2.5\mu\text{M}$) significantly increased $[\text{Ca}^{2+}]_i$, decreased $[\text{ATP}]_i$, decreased forward scatter, triggered annexin-V-binding and elicited hemolysis. Decreased $[\text{ATP}]_i$ by glucose depletion for 48 hours was similarly followed by increased $[\text{Ca}^{2+}]_i$, decreased forward scatter and annexin-V-binding. Annexin-V-binding was blunted by Ca^{2+} -removal, by the cation channel inhibitor amiloride (1mM), by the protein kinase C inhibitor staurosporine (500nM) but not by the pancaspase inhibitor zVAD (10 μM). A 48 hour treatment of erythrocytes with ochratoxin A was followed by significant increase of Fluo3-fluorescence_i ($\geq 2.5 \mu\text{M}$), increase of ceramide abundance (10 μM), decrease of forward scatter ($\geq 5 \mu\text{M}$) and increase of annexin-V-binding ($\geq 2.5 \mu\text{M}$). Ochratoxin A exposure slightly but significantly enhanced hemolysis (10 μM). Ochratoxin (10 μM) enhanced erythrocyte adhesion to HUVEC. Removal of extracellular Ca^{2+} significantly blunted, but did not abrogate ochratoxin A-induced annexin V binding. Similar to enniatin A and ochratoxin A, a 48 h treatment of erythrocytes with zearalenone ($\geq 25 \mu\text{M}$) was resulted into significant increase of $[\text{Ca}^{2+}]_i$, significant decrease of forward scatter, and significant increase of annexin-V-binding. The effect on annexin V binding was significantly blunted in the nominal absence of extracellular Ca^{2+} . Zearalenone stimulates the suicidal erythrocyte death, an effect at least partially due to stimulation of Ca^{2+} entry. The present findings show that, all three mycotoxin (enniatin A, ochratoxin A and zearalenone) which were previously reported to induce apoptotic cell death in nucleated cells, also under their *in vivo* plasma concentrations act as potent stimulators of suicidal death of erythrocytes despite the absence of gene expression and mitochondria.

The second part of the study explored the mechanisms involved in the eryptosis induced by therapeutically important phytochemicals (withaferin A, oridonin and dicoumarol). For 48 hour, erythrocytes were exposed to the three different phytochemicals with indicated concentrations leveling the range of their *in vivo* plasma levels. Withaferin A significantly decreased forward scatter (at $\geq 10 \mu\text{M}$ withaferin concentration) and increased $[\text{Ca}^{2+}]_i$ ($\geq 5 \mu\text{M}$), ROS-formation ($\geq 10 \mu\text{M}$) ceramide-formation ($\geq 10 \mu\text{M}$) as well as annexin-V-binding ($\geq 5 \mu\text{M}$). Withaferin A treatment was followed by slight but significant increase of hemolysis. Extracellular Ca^{2+} removal, amiloride, and the antioxidant N-acetyl-L-cysteine significantly blunted withaferin A-triggered annexin-V-binding. Erythrocytes were exposed to oridonin. At concentration ($\geq 25 \mu\text{M}$) oridonin significantly increased cytosolic Ca^{2+} -concentration, increased ceramide formation, decreased forward scatter and triggered annexin V-binding (the latter in $>20\%$ of the erythrocytes). Oridonin did not decrease ATP concentration and hemolysed $<5\%$ of erythrocytes. The effects of oridonin on annexin V binding were partially reversed in the nominal absence of Ca^{2+} and by the addition of amiloride (1mM). Dicoumarol ($\geq 10 \mu\text{M}$) after incubation, significantly increased $[\text{Ca}^{2+}]_i$, enhanced cation channel activity, decreased forward scatter, triggered annexin-V-binding and elicited hemolysis. Following exposure to $30 \mu\text{M}$ dicoumarol, annexin-V-binding affected approximately 15%, and hemolysis 2% of treated erythrocytes. The stimulation of annexin-V-binding by dicoumarol was abrogated in the nominal absence of Ca^{2+} . Collectively the data obtained from these studies reveal a completely novel effect of medicinally important phytochemicals (withaferin A, oridonin and dicoumarol) i.e the regulation of calcium-dependent suicidal death erythrocyte death.

3. ZUSAMMENFASSUNG

Erythrozyten können, ähnlich wie zellkernhaltige Zellen, in einen suizidalen Zelltod eintreten. Dieser Vorgang wird auch als „Eryptose“ bezeichnet. Die Mechanismen, die an der Eryptose beteiligt sind, hängen unter anderem von der Aktivierung Ca^{2+} abhängiger Kationenkanäle ab, was zu einer erhöhten intrazellulären Ca^{2+} -Aktivität führt. Eine erhöhte cytosolische Ca^{2+} -Konzentration stimuliert die Umlagerung von Phosphatidylserin an die Zelloberfläche und führt zum Zellschrumpfen. Die Eryptose ist ein physiologischer Prozess, der dazu beiträgt, dass das Überleben beschädigter Erythrozyten limitiert wird. Eine verstärkte Eryptose aufgrund von Stress, toxischer Substanzen, Krankheiten oder nicht kompensatorischen Mechanismen können zu Anämie führen. Die hier präsentierten Beobachtungen zeigen die Rolle von Xenobiotika in der Regulation der Eryptose sowie deren toxische Effekte auf Erythrozyten.

Der erste Teil der Studie untersuchte die Mechanismen verschiedener in Lebensmitteln vorhandenen Mycotoxinen (Enniatin A, Ochratoxin A und Zearalenon), die eine Eryptose auslösen. Die Inkubation von Erythrozyten für 48 Stunden mit Enniatin A ($\geq 2.5 \mu\text{M}$) erhöhte signifikant die intrazelluläre Ca^{2+} -Konzentration und die Annexin-V-Bindung, erniedrigte signifikant [ATP] und den Forward Scatter und löste Hämolyse aus. Ein erniedrigter ATP-Gehalt nach Glukose-Depletion führte in ähnlicher Weise zu einer erhöhten Ca^{2+} -Konzentration, erniedrigte das Forward Scatter und erhöhte die Annexin-V-Bindung. Die erhöhte Anzahl an Annexin-V-positiven Erythrozyten wurde durch das Entfernen von Ca^{2+} , durch den Kationen-Kanal Inhibitor Amilorid (1mM) und durch den Protein-Kinase C Inhibitor Staurosporin (500 nM) verringert, jedoch nicht durch den Pancaspase Inhibitor zVAD (10 μM). Eine 48 stündige Behandlung der Erythrozyten mit Ochratoxin A hatte eine erhöhte Fluo3-Fluoreszenz, einen erhöhten Ceramid-Gehalt (10 μM), ein erniedrigtes Forward Scatter ($\geq 5 \mu\text{M}$) und eine erhöhte Annexin-V-Bindung ($\geq 2.5 \mu\text{M}$) zur Folge. Die Ochratoxin A-Exposition erhöhte schwach, aber dennoch signifikant, die Hämolyse (10 μM). Ochratoxin A (10 μM) erhöhte die Adhäsion der Erythrozyten an HUVEC-Zellen. Das Entfernen von extrazellulären Ca^{2+} -Ionen erniedrigte zwar die mittels durch Ochratoxin A induzierte Annexin-V-Bindung, hebte den Effekt jedoch nicht auf. Ähnlich wie Enniatin A und Ochratoxin A, führte eine 48 stündige Inkubation der Erythrozyten mit Zearalenon ($\geq 25 \mu\text{M}$) zu einer erhöhten intrazellulären Ca^{2+} -Konzentration, zu einem erniedrigten Forward Scatter und zu einer signifikant erhöhten Annexin-V-Bindung. Der Effekt

auf die Annexin-V-Bindung wurde durch das Entfernen der extrazellulären Ca^{2+} -Ionen signifikant abgeschwächt. Zearalenon stimuliert den suizidalen Erythrozytentod, ein Effekt, der zum Teil auf die Stimulation des Ca^{2+} -Einstroms zurückzuführen ist.

Die gegenwärtigen Beobachtungen zeigen, dass die drei Mycotoxine Enniatin A, Ochratoxin A und Zearalenon, von denen bekannt ist, dass sie den apoptotischen Zelltod in zellkernhaltigen Zellen auslösen, mit Konzentrationen im Bereich der in vivo gefundenen Plasmakonzentrationen auch als Stimulatoren des suizidalen Erythrozytentods fungieren, obwohl diese Zellen weder eine Genexpression noch Mitochondrien aufweisen.

Der zweite Teil der Studie untersuchte die Mechanismen, die an der Eryptose durch die therapeutisch wirksamen Phytochemikalien Withaferin A, Oridonin und Dicoumarol beteiligt sind. Erythrozyten wurden für 48 Stunden den drei verschiedenen Phytochemikalien mit jeweiligen Konzentrationen, die sich im Bereich der Plasmakonzentrationen befanden, ausgesetzt. Withaferin A erniedrigte signifikant den Forward Scatter ($\geq 10 \mu\text{M}$) und erhöhte $[\text{Ca}^{2+}]_i$ ($\geq 5 \mu\text{M}$), sowie die Bildung von ROS ($\geq 10 \mu\text{M}$), die Ceramid-Bildung ($\geq 10 \mu\text{M}$) und die Annexin-V-Bindung ($\geq 5 \mu\text{M}$). Die Withaferin-Behandlung hatte eine schwache, aber signifikante Hämolyse zur Folge. Das Entfernen von extrazellulärem Ca^{2+} , die Behandlung mit Amilorid und dem Antioxidans N-Acetyl-L-Cystein schwächte signifikant die Withaferin A- induzierte Annexin-V-Bindung ab.

Erythrozyten, die mit Oridonin behandelt wurden, zeigten bei einer Konzentration von $\geq 25 \mu\text{M}$ Oridonin eine signifikant erhöhte cytosolische Ca^{2+} -Konzentration, verstärkte Ceramid-Bildung, ein vermindertes Forward Scatter und vermehrte Annexin-V-Bindung (in $>20\%$ der Erythrozyten). Oridonin erhöhte nicht die ATP-Konzentration und hämolysierte $<5\%$ der Erythrozyten. Der Effekt von Oridonin auf die Annexin-V-Bindung wurde teilweise durch das Entfernen von Ca^{2+} aus der Inkubationslösung und durch die Zugabe von Amilorid (1mM) aufgehoben. Dicoumarol ($\geq 10 \mu\text{M}$) erhöhte nach Inkubation signifikant die Ca^{2+} -Konzentration sowie die Kationenkanal-Aktivität, erniedrigte das Forward Scatter und stimulierte die Annexin-V-Bindung sowie die Hämolyse. Nach der Behandlung der Erythrozyten mit $30 \mu\text{M}$ Dicoumarol betrug die Annexin-V-Bindung ungefähr 15% und die Hämolyse 2% . Die Stimulation der Annexin-V-Bindung durch Dicoumarol wurde durch das Entfernen von extrazellulären Ca^{2+} -Ionen abgeschwächt. Zusammengefasst zeigen die Daten dieser Studien einen neuartigen Effekt

medizinisch wichtiger Phytochemikalien, namentlich Withaferin A, Oridonin und Dicoumarol,,
wie z.B. die Regulation des von Calcium abhängigen suizidalen Erythrozytentods.

4. INTRODUCTION

4.1. Erythrocytes

Erythrocytes are considered to be physiologically important and the most abundant cells in the human body and are mentioned in the literature as RBCs, red cells, red blood corpuscles, haematids and erythroid cells. Erythrocytes were for the first time identified by the Dutch biologist Jan Swammerdam, in 1658. Adult humans have approximately $2-3 \times 10^{13}$ (20-30 trillion) red blood cells which are about one quarter of the total cells in the body (Foller, Huber et al. 2008). Women have about 4 to 5 million and men about 5 to 6 million erythrocytes per μL of blood with exception of people living at high altitudes with low oxygen tension will have more. Red blood cells are more common than the other blood cells, in 1 μL of normal human blood there are about 4,000–11,000 white blood cells and 150,000–400,000 platelets (Pierige, Serafini et al. 2008). Erythrocytes have iron containing protein hemoglobin, which is principally responsible for transportation of O_2 and CO_2 in the body. Due to lack of nucleus and mitochondria, all the intracellular space in erythrocyte is available for gaseous transport (Tavassoli 1978).

The formation of erythrocyte or erythropoiesis is stimulated in bone marrow by the renal hormone erythropoietin (EPO). Erythrocytes circulate in the blood for about 100–120 days before they are engulfed and their components are recycled by macrophages. Normal time for each circulation is about 20 seconds. Approximately 25 % of the cells in the human body comprises of red blood cells (Pierige, Serafini et al. 2008).

The diameter and thickness of a typical human erythrocyte is 6–8 μm and 2 μm respectively, smaller in size than most other human cells, having average volume of about 90 fL (Goodman, Kurdia et al. 2007). The red color of erythrocytes is due to the spectral characteristics of the hemic iron ions in hemoglobin. Normal erythrocyte contains approximately 270 million of hemoglobin biomolecules and there are four heme groups in each molecule. Hemoglobin occupies about a third of the total cell. 98% of the oxygen transported to other body tissue by binding with hemoglobin and the remaining about 2% oxygen dissolved in the blood plasma and carried to the tissue. The erythrocytes of average adult human contain about 2.5 grams of iron

which is about 65% of the total iron stores of contained in the body (McLaren, Brittenham et al. 1987)

4.2. Erythropoiesis

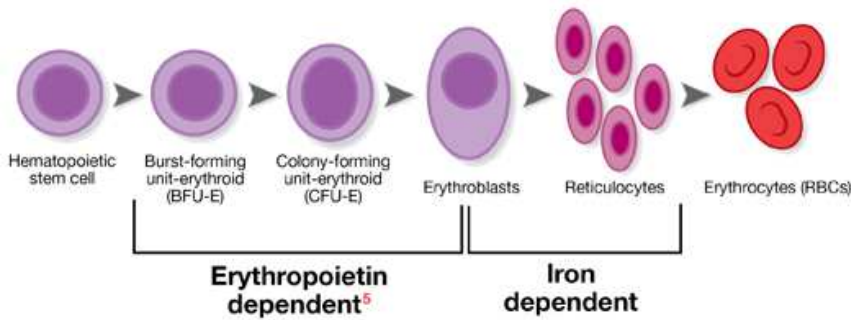


Figure 1. Erythropoiesis (Bron, Meuleman et al. 2001).

The process by which erythrocytes are generated in the body is known as erythropoiesis (Figure 1). The whole process of development from precursor stem cells to mature erythrocytes comprises of about 7 days. It is induced by decreased O_2 in circulation, at that point which the kidneys secrete erythropoietin. This hormone mediates proliferation and differentiation of red cell precursors, which results in activation of erythropoiesis in the hemopoietic tissues. In mammals (including humans), erythrocyte production usually occurs within the bone marrow but there are a few exceptions. In early fetal life, erythropoiesis takes place in the mesodermal cells of the yolk sac. In third or fourth month, spleen and liver are responsible for erythropoiesis (Palis and Segel 1998). After seven months, erythropoiesis starts normally in the bone marrow. Mature cells circulate for about 100 to 120 days in the body. At the end of their life span, the old erythrocyte undergoes some structural changes in its plasma membrane, which make it susceptible for macrophages and subsequently phagocytosis in the reticuloendothelial system (Foller, Huber et al. 2008). Adopting this way, the old and defective cells are continually removed from the blood without hemolysis.

4.3. Anemia

Anemia is a pathological condition in which, there is less number of healthy red blood cells or less quantity of hemoglobin than normal in blood (Walters and Abelson 1996). However, it can include decreased oxygen-binding ability of each hemoglobin molecule due to deformity or lack

in numerical development. Hemoglobin normally carries oxygen from the lungs to the capillaries, anemia may lead to hypoxia in organs. As all human cells need oxygen for proper function, varying degrees of anemia may lead to a wide range of clinical consequences.

Anemia is one of the most common disorders of the blood. Classification of anemia can be done by a variety of approaches. On the basis of severity, Anemia may be mild, moderate or severe. In mild anemia, hemoglobin ranges from 9.5 to 11 g/dl. Mild anemia is an asymptomatic condition. In moderate anemia, hemoglobin ranges from 8 to 9.5 g/dl, may present with other symptoms but need proper management to prevent complications. In severe anemia, hemoglobin concentration is always less than 8 g/dl. This is symptomatic and needs prompt attention to avoid severe complications. Anemia can be life threatening, depends upon the causes and magnitude of the RBC and hemoglobin deficiency (Segel, Hirsh et al. 2002).

On the basis of erythrocytic morphology, anemia can be classified as macrocytic (MCV >100), normocytic (MCV=100) and microcytic (MCV<80). The bases for etiological classification of anemia include hemorrhage, hemolysis and defective erythropoiesis. Most common symptoms of anemia include weakness, fatigue, malaise, poor concentration, pale skin, shortness of breath, dizziness and restless legs syndrome.

To remain functional in blood stream, erythrocytes size and shape should be maintained. Erythrocyte with abnormal shapes like spherocytes or elliptocytes and abnormal sizes like microcytes or macrocytes may not remain active in the bloodstream for the full life span. If sufficient premature destruction occurs, then there is a chance of disturbance between the production and destruction of erythrocytes (Salsbury 2001) which may lead to anemia.

4.4. Erythrocyte membrane composition

The erythrocyte membrane plays a vital role in the regulation of erythrocyte surface deformability, flexibility, adhesion to other cells and immune recognition. These properties are completely dependent on the composition of the membrane.

The erythrocyte membrane is composed of 3 layers, the exterior carbohydrate-rich glycocalyx, the lipid bilayer which contains many transmembrane proteins, and a structural network of proteins located on the inner surface of the lipid bilayer also membrane skeleton. By mass, half of the cell membrane portion in human erythrocytes is proteins. The other half are lipids,

namely phospholipids and cholesterol (Yazdanbakhsh, Lomas-Francis et al. 2000). The phospholipids are asymmetrically embedded in the bilayer (Steck 1974). The outer side of the bilayer contains sphingomyelin, glycolipids and phosphatidylcholine. The inner part contains phosphatidylinositol, phosphatidylserine and phosphatidyl-ethanolamine. (Smith 1987, Palek 1993).

The red cell membrane skeleton is a group of protein complexes formed by structural proteins including α and β spectrin, ankyrin, protein 4.1 and actin. The key role of the proteins is to interact with the lipid bilayer and also with transmembrane proteins to keep the cell membrane intact (Tse and Lux 1999). So far, more than fifty erythrocyte membrane proteins have been recognized and majority of them related to various blood group antigens. Functionally, some of the important membrane proteins are including band 3, Glut 1, Kidd antigen protein, aquaporin 1, Gardos channel, $\text{Na}^+\text{-K}^+\text{-ATPase}$, $\text{Ca}^{2+}\text{-ATPase}$, $\text{Na}^+\text{-K}^+\text{-2Cl-cotransporter}$, $\text{Na}^+\text{-K}^+$ cotransporter, $\text{K}^+\text{-Cl-cotransporter}$, $\text{Na}^+\text{-Cl-cotransporter}$, ICAM-4 and laminin.

Additionally, membranous proteins are responsible for the adhesion, deformability, flexibility and durability. All mentioned properties enable erythrocytes to squeeze through capillaries up to their less than half of the diameter (7-8 μm) and regaining of the discoid shape at the end of circulation through capillaries.

4.5. Ionic transportation in Erythrocyte

Erythrocytes are different from other body cells as they lack both nucleus and mitochondria. Erythrocyte further contains an iron containing protein called hemoglobin. This protein has a vital role in the balance of ionic environment of the cell by oxygen and carbon dioxide transport and pH maintenance in the blood. Additionally, it has role in the vasodilatation of arterioles due to binding affinity with nitric oxide (Bessis and Delpech 1981). As shown in the figure 2, ionic transportation in erythrocytes mainly done by $\text{Ca}^{2+}\text{-ATPase}$, $\text{Na}^+\text{-K}^+\text{-ATPase}$, Gardos channel, $\text{K}^+\text{/Cl}^-$ co-transport, Band 3 anion exchanger $\text{Na}^+\text{/K}^+\text{/2Cl}$ co-transporter and glucose transporter (Maher and Kuchel 2003)

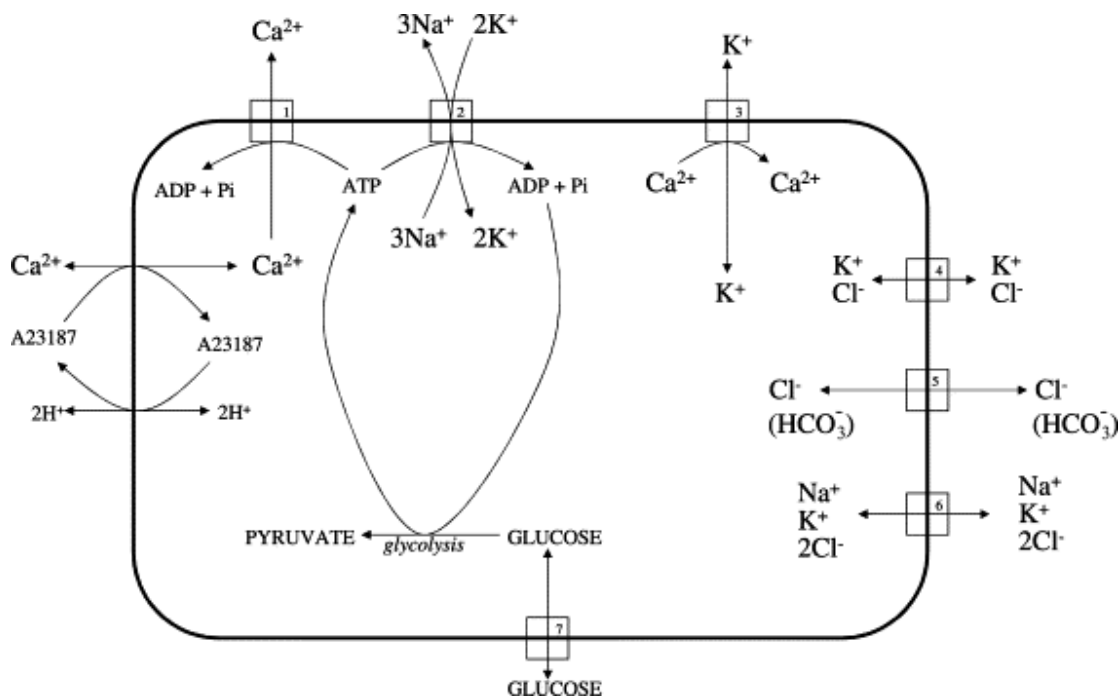


Figure 2. Ion transportation mechanism in the erythrocyte membrane (Maher and Kuchel 2003).

The Ca^{2+} -ATP-ase proteins in erythrocyte are responsible for movement of cytosolic Ca^{2+} from intra to the extra-cellular space, mainly for the regulation of signaling inside the cell. Signaling by these transmembrane proteins has key role in the apoptosis of erythrocytes. Any defective or inhibition of the Ca^{2+} -ATPase develops high cytosolic Ca^{2+} concentration and could cause apoptosis of erythrocytes also known as eryptosis (Lang, Huber et al. 2007).

The transmembrane Na^+/K^+ -ATP-ase channels of erythrocytes involved in the regulation of the Na^+ and K^+ concentrations inside the erythrocyte. It is ATP-dependent pump. Normal Na^+ , K^+ flux ratio maintain by this pump in erythrocytes is 3:2 (Lew and Bookchin 2005).

Gardos channels are Ca^{2+} sensitive K^+ channels in erythrocytes. These channels play a vital role in the regulation of suicidal erythrocyte death (Lang, Kaiser et al. 2003, Ghashghaeinia, Cluitmans et al. 2012). About 100-200 Gardos channels are located on red blood cell membrane (Lew, Muallem et al. 1982, Alvarez and Garcia-Sancho 1987). Gardos channels remain inactive, at 20-50nM and activates at 150 nM-2 μM Ca^{2+} concentration (Lew and Bookchin 2005). The principal out come of the activation of Gardos channels is the release of K^+ and Cl^- from the cell. There is parallel loss of water which leads to erythrocyte shrinkage (Foller, Huber et al. 2008).

KCl cotransporters and Band3 (AE1) proteins are abundant and highly active in precursor's cells of erythrocytes. In reticulocytes, the passive K^+ fluxes are regulated by the KCl cotransporter (Hall and Ellory 1986). In developing erythroblasts, Band3 (AE1) plays a major role as an integral protein for the assembly of the membrane skeleton (Peters, Shivdasani et al. 1996).

4.6. Apoptosis

Apoptosis is the term alternatively used for programmed or suicidal cell death, characterized by morphological changes and mediated by energy-based biochemical mechanisms (Elmore 2007). The term apoptosis was used for the first time by Kerr, Wyllie, and Currie in 1972, although various components of the programmed erythrocyte death had been described many years before (Paweletz 2001, Kerr 2002). In comparison to apoptosis, necrosis is a kind of traumatic cell death, generally because of acute cellular injury. Physiologically, apoptosis is recognized as a homeostatic mechanism during development and aging stages, mainly to maintain the normal populations of cell in tissues. Similarly, it acts as a defense mechanism in the body (Norbury and Hickson 2001).

The morphological changes occurs during apoptosis includes membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and DNA fragmentation. At the end apoptotic cell bodies are phagocytosed by macrophages without any inflammatory process (Savill and Fadok 2000, Kurosaka, Takahashi et al. 2003).

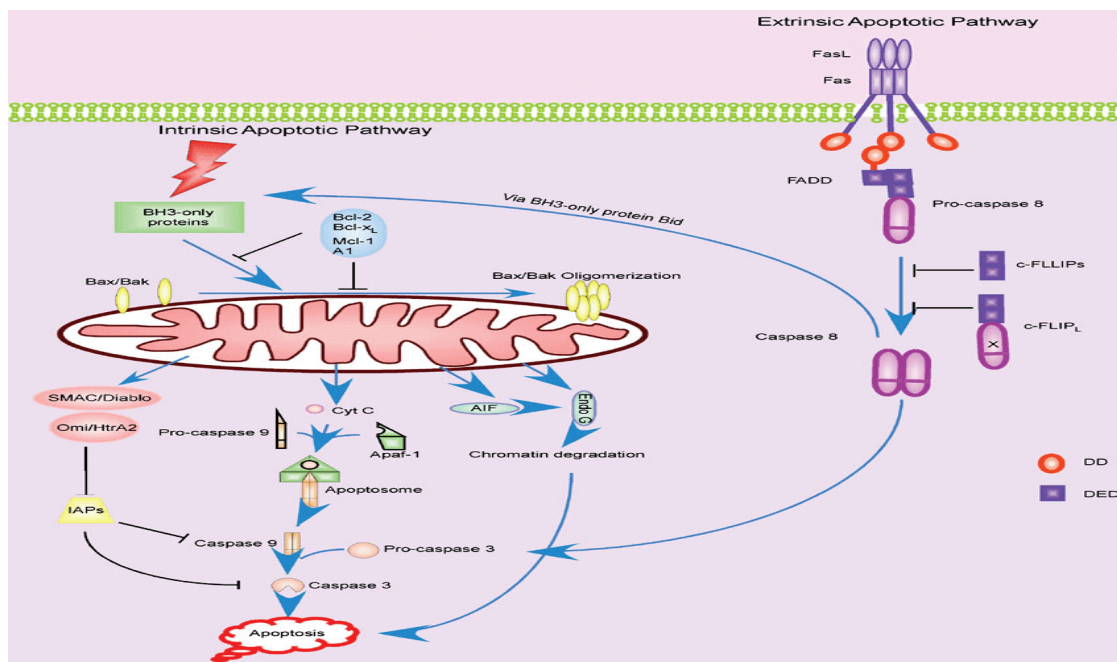


Figure 3. Mechanisms of apoptosis (Zhang, Hartig et al. 2005)

There are two main apoptotic pathways, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Figure 3). Both pathways are linked together (Igney and Krammer 2002). Perforin/granzyme pathway recognized as an additional pathway, involving T-cell-mediated cytotoxicity and perforin-granzyme-dependent suicidal death of cell (Elmore 2007). The cytotoxic T lymphocytes (CTLs) kill antigen bearing cells via the extrinsic pathway. The FasL/FasR interaction is the key way of CTL mediated apoptosis (Brunner, Wasem et al. 2003). All pathways finally converge on an execution phase.

In extrinsic pathway, death receptors from tumor necrosis factor (TNF) receptor family are the key players (Locksley, Killeen et al. 2001). These receptors have cyteine-rich extracellular domains with a cytoplasmic domain that is comprises of 80 amino acids known as death domain (Ashkenazi and Dixit 1998). This death domain has a vital contribution in the transmission of the signal from the surface of the cell to the intracellular signaling pathways. In extrinsic pathway, the recognized ligands and their corresponding death receptors are TNF- α /TNFR1, FasL/FasR, Apo3L/DR3, Apo2L/ DR4 and Apo2L/DR5 (Suliman, Lam et al. 2001, Rubio-Moscardo, Blesa et al. 2005).

In contrast, the intrinsic pathways initiate apoptosis by non-receptor-mediated stimuli. The intracellular signals directly act on mitochondria and cause changes in the inner membrane. The consequences of this phenomenon includes, opening of the mitochondrial permeability transition (MPT) pore, loss of transmembrane potential and release two groups of intermembrane pro-apoptotic proteins (Saelens, Festjens et al. 2004). The first group proteins are, cytochrome c, Smac/DIABLO and the serine protease HtrA2/Omi (Garrido, Galluzzi et al. 2006). These proteins form an apoptosome by activating caspase-dependent mitochondrial pathway. The second group of pro-apoptotic proteins comprises of AIF, endonuclease G and CAD. These are released by mitochondria at late stage of apoptosis. AIF causes DNA fragmentation and condensation of nuclear chromatin (Joza, Susin et al. 2001). All these apoptotic mitochondrial events are regulated by Bcl-2 proteins family (Cory and Adams 2002).

4.7. Eryptosis

Eryptosis is the suicidal death of erythrocytes. Aged erythrocytes undergo senescence which results in their clearance from the body. Physiologically, eryptosis limits the erythrocyte life span to approximately 100–120 days. Events take places for erythrocyte senescence includes, attachment of hemichromes to band 3, cluster formation of band 3 and deposition of C3 complement fragments and anti-band 3 immunoglobulins (Lang and Qadri 2012).

Programmed cell death has a vital role in the removal of abundant, damaged or potentially harmful cells (Gulbins, Jekle et al. 2000). Similar to nucleated cells, erythrocytes are cleared from the circulation by suicidal death. As shown in the figure. 4, the macrophages speedily engulf and destroy phosphatidylserine exposing cells (Boas, Forman et al. 1998).

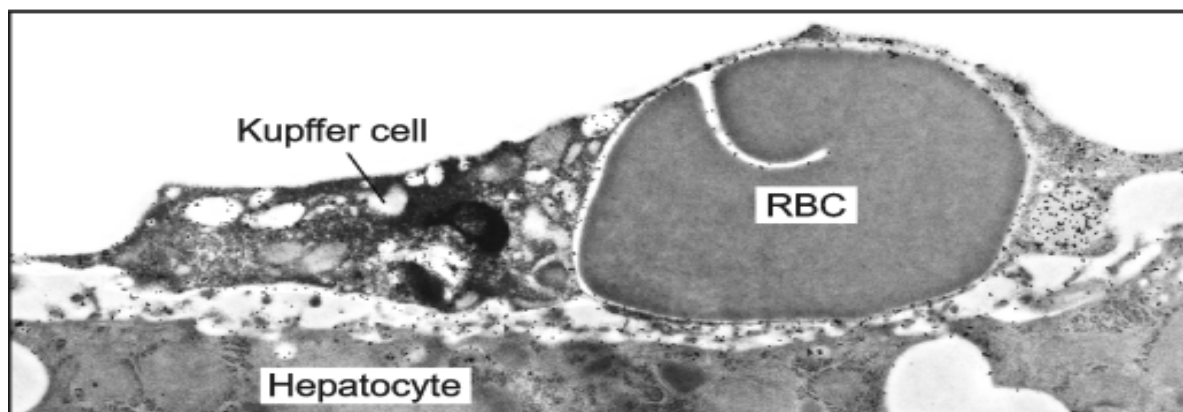


Figure 4. Phagocytosis of phosphatidylserine exposing erythrocyte by a Kupffer cell in rat liver (Lang, Lang et al. 2005)

Structurally, erythrocytes are deficient of important organelles like nuclei and mitochondria, which are considered as key players in apoptosis. Due to this, erythrocytes lack events of apoptosis like mitochondrial depolarization, nuclear condensation and DNA fragmentation. However, eryptosis possesses similar features of apoptosis including loss of cellular K^+ along with cell shrinkage, membrane blebbing and translocation of phosphatidylserine in the plasma membrane (Bortner and Cidlowski 1999).

Due to structural differences, aged erythrocytes eliminated from the circulation by different mechanisms in comparison to apoptosis of nucleated cells. Ca^{2+} is most important element in the execution of eryptosis. Incubation of erythrocytes with the Ca^{2+} ionophore ionomycin bring changes like cell shrinkage, membrane blebbing and phosphatidylserine exposure in the cell (Berg, Engels et al. 2001). The cell shrinkage is due to loss of KCl , by the activation of the Ca^{2+} sensitive K^+ channels (Lang, Kaiser et al. 2003). Similarly, phospholipid scrambling of the cell membrane, may be because of activation of a Ca^{2+} sensitive scramblase (Dekkers, Comfurius et al. 2002), inhibition of Ca^{2+} sensitive and ATP-dependent phospholipid translocase (Seigneuret and Devaux 1984). Due to some similarities as well as differences from apoptosis, the term eryptosis alternatively used to represent suicidal death of erythrocytes.

4.7.1. Signaling pathways of eryptosis

Ion channels of cellular membrane play a vital role in the erythrocyte suicidal death (Lang, Huber et al. 2007). Ca^{2+} -permeable cation channels and Ca^{2+} sensitive K^+ channels (Gardos channels) are key player in signaling of eryptosis.

High cytosolic Ca^{2+} concentration could be the main cause of eryptosis (Figure 5) and may lead to cell membrane scrambling and phosphatidylserine exposure (Akel, Hermle et al. 2006, Nicolay, Schneider et al. 2006). Further, Ca^{2+} entry triggers an endopeptidase calpain, which facilitates cell membrane blebbing by degradation of the erythrocyte cytoskeleton (Pant, Virmani et al. 1983). Nonselective cation channels are responsible Ca^{2+} transport to the cytosol (Kaestner and Bernhardt 2002, Lang, Kempe et al. 2005). The activation of these channels is principally triggered by osmotic shock (Huber, Gamper et al. 2001), oxidative stress (Duranton, Huber et al. 2002) and Cl^- removal (Lang, Kempe et al. 2005)

Another important effect of high erythrocyte cytosolic Ca^{2+} is the stimulation of Ca^{2+} -sensitive K^+ channels. (Bookchin, Ortiz et al. 1987). (Figure 5). Activation of Ca^{2+} -sensitive K^+ channels leads to the Cl^- removal along with K^+ (Lang, Warskulat et al. 2003). The loss of KCl parallel with water from the cell causes erythrocyte cell shrinkage (Lang, Warskulat et al. 2003).

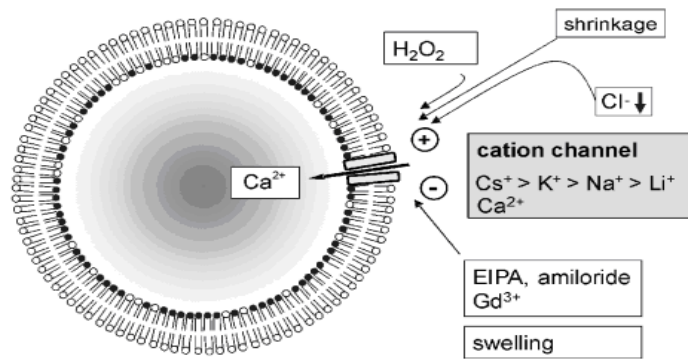


Figure 5. Cation channel regulation in eryptosis (Lang, Lang et al. 2005)

Ceramide is an important factor to enhance the Ca^{2+} sensitivity of cellular membrane scrambling, similar to elevated intracellular Ca^{2+} levels which elicits phosphatidylserine translocation in the membrane (Lang, Myssina et al. 2004). Ceramide formation is under controlled by platelet activating factor. PAF triggers sphingomyelinase, which facilitate sphingomyelin breakdown

(Figure 6) (Lang, Kempe et al. 2005). PAF-mediated eryptosis is considered as Ca^{2+} -independent eryptosis, as high Ca^{2+} concentration does not compulsory for PAF activation (Lang, Kempe et al. 2005). Further, PAF sensitize Ca^{2+} -sensitive K^+ channels to stimulate the effects of intracellular Ca^{2+} (Rivera, Jarolim et al. 2002).

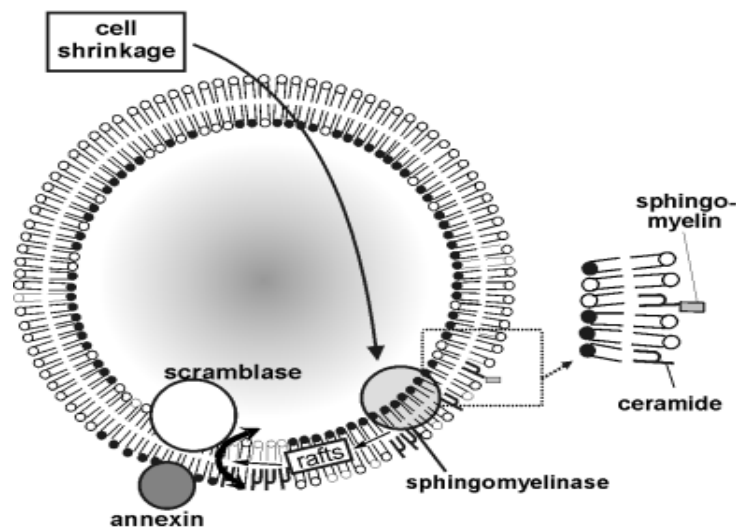


Figure 6. Regulation of sphingomyelinase in eryptosis (Lang, Lang et al. 2005)

Energy-depleted environment is among the stimulators of eryptosis. Figure 7 shows the mechanism involved in eryptosis induced by energy depletion which is due to the activation of protein kinase C (PKC) and PKC-dependent phosphorylation of cytoskeletal proteins (Figure 7) (Klarl, Lang et al. 2006). Erythrocytes express PKCa, PKCi, PKCl and PKCf (Govekar and Zingde 2001). PKC activation leads to triggering of Ca^{2+} entry (Andrews, Yang et al. 2002), phosphatidylserine exposure (de Jong, Rettig et al. 2002).

Oxidative stress or defective anti-oxidative defense (Bilmen, Aksu et al. 2001) stimulate eryptosis by facilitating Ca^{2+} entry through the activation of the Ca^{2+} -permeable cation channels (Duranton, Huber et al. 2002). Ca^{2+} -permeable cation channels activity leads to erythrocyte shrinkage and thus become the source of induction of eryptosis (Myssina, Lang et al. 2004). Figure 7 gives diagrammatic description of the mechanism involved in eryptosis triggered by oxidative stress. Oxidative stress mediated suicidal erythrocyte death is paralleled by the activation of aspartyl and cysteinyl proteases (Mandal, Baudin-Creuzat et al. 2003). During oxidative stress, erythrocytes expressed caspases (Bratosin, Estaquier et al. 2001), which leads to the cleavage of the anion exchanger band 3 (Mandal, Baudin-Creuzat et al. 2003). All events

results in the induction of phosphatidylserine translocation in the membrane (Mandal, Moitra et al. 2002).

Hyper osmotic shock-induced eryptosis is mediated by two clear signaling mechanisms (Figure 7). In the first mechanism, there is an activation of cyclooxygenase (COX), which leads to the release of prostaglandin E₂ (Lang, Kempe et al. 2005). PGE₂ activates Ca²⁺ permeable cation channels (Kaestner and Bernhardt 2002), subsequently increases the intracellular Ca²⁺ activity and phosphatidylserine exposure on the erythrocyte membrane (Kaestner, Tabellion et al. 2004). Secondly, osmotic shock turns on phospholipase A₂, which regulates the release of platelet activating factor. PAF triggers ceramide production by activation of sphingomyelinase in erythrocytes (Lang, Kempe et al. 2005).

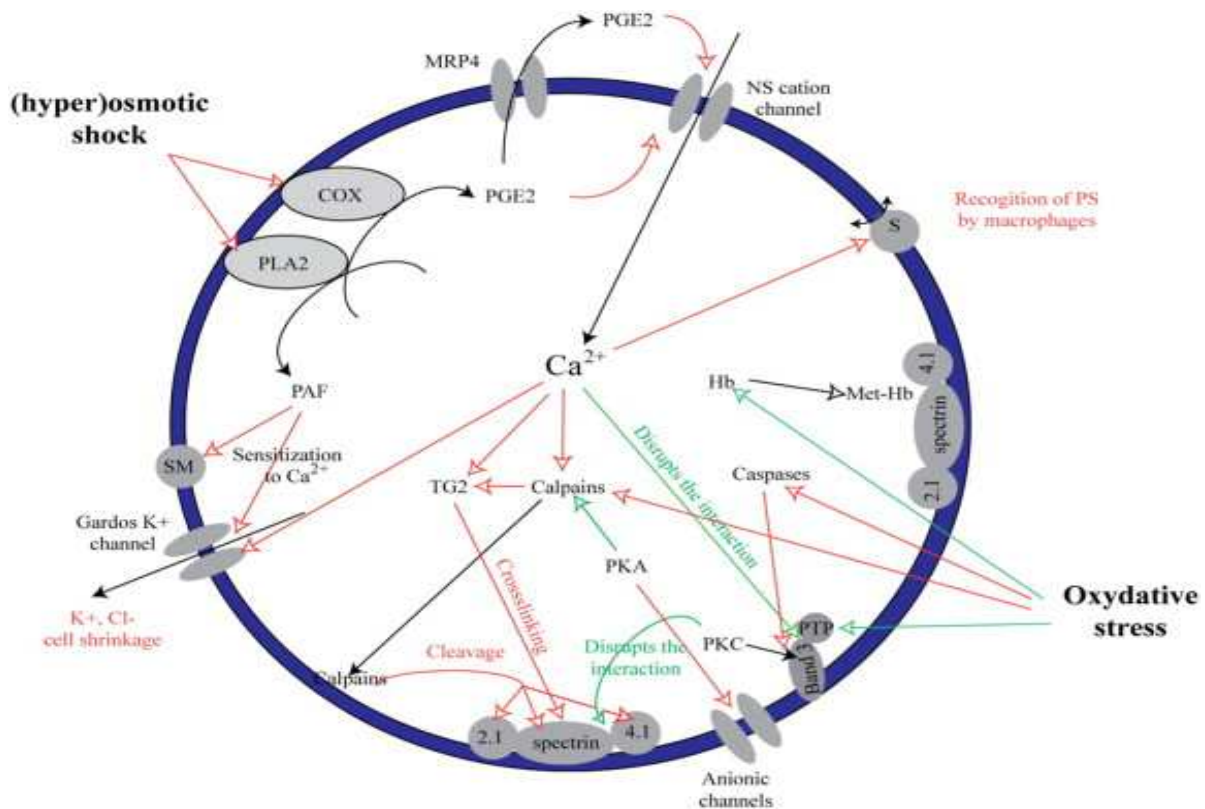


Figure 7. Summary of suicidal erythrocyte death signaling (Lion, Crettaz et al. 2010).

4.7.2. Eryptosis inducers

Eryptosis may be speeded up by stress conditions like osmotic shock (Lang, Duranton et al. 2003), oxidative stress and energy depletion (Lang, Roll et al. 2002). Some other inducers of eryptosis includes aluminium, amantadine, amiodarone, amphotericin B, amyloid, anandamide, anti-A IgG, arsenic, azathioprine, bay 11-7082, bay-Y5884, beauvericin, bismuth chloride, cadmium, ceramide (acylsphingosine), chlorpromazine, ciglitazone, cisplatin, copper, cordycepin, curcumin, cyclosporine, CD95/Fas/ligand, dimethylfumarate, FTY720, glycophorin-C, gold salts, hemin, hemolysin, IPA3, lead, leukotriene C, lipopeptides, listeriolysin, lithium, mercury, methyl dopa, methylglyoxal, monensin, paclitaxel, PAF, parthenolide, peptidoglycan, phytic acid, prostaglandin E₂, radio contrast agent, retinoic acid, selenium, silver ions, thrombospondin-1, receptor CD47, thymoquinone, tin, valinomycin, sodium vanadate, sphingosine, vitamin K(3), zinc (Lang and Qadri 2012)

4.7.3. Diseases associated with enhanced eryptosis

Suicidal erythrocyte death is an important feature of many diseases. The diseases showing eryptosis are Iron deficiency phosphate depletion, neocytolysis, sepsis, hemolytic anemia, hemolytic uremic syndrome, renal insufficiency, diabetes, malaria, sickle-cell disease, thalassemia, G6PD deficiency, Wilson's disease, AE1 mutation, GLUT1 mutation, paroxysmal nocturnal hemoglobinuria, myelodysplastic syndrome, hereditary spherocytosis, mycoplasma infection, hyperthermia (Lang and Qadri 2012).

The mouse models with sickle cell anemia, thalassemia, annexin A7 deficiency, cGKI deficiency, AMPK deficiency, Endothelin B receptor deficiency, AE1 deficiency, APC deficiency, Klotho deficiency, EPO excess has increasing eryptosis tendency (Lang and Qadri 2012).

4.7.4. Inhibitors of eryptosis

Certain drugs and natural products are reported as an inhibitor for suicidal death of erythrocytes or eryptosis. The important inhibitors for eryptosis are adenosine, amitriptyline, blebbistatin, caffeine, catecholamines (isoproterenol), chloride, EIPA, endothelin 1, EPO, flufenamic acid, NBQX/CNQX, niflumic acid, NO (nitroprusside), NPPB, phlorhizin, resveratrol, sarafotoxin 6c, staurosporine, thymol, urea, WHI-P131/JANEX-1, WHI-P154, xanthohumol, zidovudine. (Lang

and Qadri 2012). The mouse model with reduce eryptosis are PDK1 deficiency, TRPC6 deficiency, PAF receptor deficiency, JAK3 deficiency (Lang and Qadri 2012)

4.7.5. Consequences related to eryptosis

Suicidal erythrocyte death is a physiological process but excessive eryptosis due to stress, toxicity, diseases or defective compensatory mechanism may leads to anemia. Excessive hemolysis due to cell swelling can be prevented, as phosphatidylserine exposure at the cell surface of injured erythrocyte is recognized by macrophages, which facilitate clearance of erythrocytes from the circulation prior to hemolysis. Further, the activation of Ca^{2+} sensitive K^+ channels resist swelling and disruption of damaged erythrocytes, thus increased the time for macrophages to clear the injured erythrocytes from the blood.

The growth of malaria pathogen *Plasmodium falciparum* can be prevented by rapid clearance of infected erythrocytes. The pathogen induces oxidative stress, which facilitate Ca^{2+} entry by the activation of Ca^{2+} - sensitive cation channels and induce eryptosis. The whole mechanism leads to the removal of the eryptotic erythrocytes by macrophages. Eventually, reduces the life span of the infected cells along with parasites (Lang, Lang et al. 2004).

Eryptotic erythrocytes have tendency to adhere with the endothelial cells of vascular wall and cause blockage in microcirculation (Lang, Gulbins et al. 2010). Due to excessive eryptosis and subsequent adherence, the stoppage in the microcirculation of kidney medulla may leads to acute renal failure (Abed, Balasaheb et al. 2013).

4.8. Xenobiotics

The term *xenobiotics* is frequently used for chemical compounds which are found in living organism, but normally these compounds are not produced and are not expected to be found in that organism. Most of the drugs are xenobiotics, as these are neither produced naturally, nor are a part of a normal diet. In other ways, xenobiotics are those substances which are found in higher concentration than normal or the artificial substances that are synthetically produced by living organism.

Xenobiotics may be either exogenous or endogenous. The exogenous xenobiotics are compound which are neither normally ingested nor utilized. They get entry therapeutically or by food

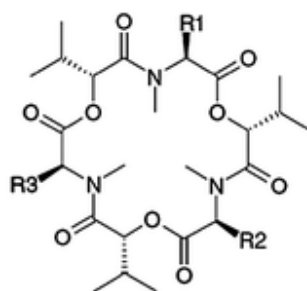
ingestion. The examples of exogenous xenobiotics are drugs, food additives, pollutants, toxins, phytochemicals, insecticide and chemical carcinogens. Endogenous xenobiotics are not foreign compounds as they are produced in the body as a metabolite in certain processes. The examples of endogenous xenobiotics are bile acids, bilirubin, steroids, eicosanoid and some fatty acids. Pharmaceutically, xenobiotics are very important. It is reported that among all newly approved drugs in past year about 40% of the newly approved drugs are either natural products or their derivatives and analogues (Cragg, Newman et al. 1997, Tietze, Bell et al. 2003).

The body removes xenobiotics by metabolism in the liver. This process comprises of the deactivation and the excretion. The routes for the excretion of xenobiotics are urine, feces, breath, and sweat. A group of liver enzymes like microsomal cytochrome P450 are responsible for the metabolism of xenobiotics. Enzyme activate xenobiotics metabolism by different ways like oxidation, reduction, hydrolysis or hydration. After that the secondary metabolite bind with glucuronic acid or glutathione and subsequently excreted in bile or urine.

4.9. Mycotoxins

Mycotoxins are toxic secondary metabolites produced by an organism from the kingdom fungi, also known as moulds (Richard 2007). Fungi are recognized source of several novel natural products. Many of these compounds are toxic but in low concentrations. They were shown to possess remarkable biological activities, so used as therapeutics in human or veterinary medicine (Firakova, Proksa et al. 2007). Mycotoxins are toxic chemicals produced by fungi that normally inhabit in food crops (Turner, Subrahmanyam et al. 2009). One type of mould can produce chemically different mycotoxin, but one mycotoxin may be produced by other fungal species (Robbins, Swenson et al. 2000). Some of the important mycotoxins includes aflatoxins,, ochratoxin A, citranin, patulin and toxins from fusarium species.

4.9.1. Enniatin A



Enniatin	R1	R2	R3
A	CH(CH ₃)CH ₂ CH ₃	CH(CH ₃)CH ₂ CH ₃	CH(CH ₃)CH ₂ CH ₃
A ₁	CH(CH ₃)CH ₂ CH ₃	CH(CH ₃)CH ₂ CH ₃	CH(CH ₃) ₂
B	CH(CH ₃) ₂	CH(CH ₃) ₂	CH(CH ₃) ₂
B ₁	CH(CH ₃) ₂	CH(CH ₃) ₂	CH(CH ₃)CH ₂ CH ₃

Figure 8. Chemical structure of enniatins (Meca, Giri et al. 2012).

Enniatins are cyclic hexadepsipeptide antibiotics (Dornetshuber, Heffeter et al. 2007). Enniatins belongs to a class of organic chemical compounds produced by *fusarium* species (Firakova, Proksa et al. 2007). While working with antibiotic-producing fungi, Gaumann discovered some fungal metabolites that were named as enniatin (Burmeister and Plattner 1987). *Fusarium* strains which are responsible for production of enniatins were isolated from different sources like walnut tree leaves (Bergendorff, Anke et al. 1994), marine algae *Codium fragile* (Jiang, Barret et al. 2002), foliage of balsam fir *Abies balsamea* (Strongman, Strunz et al. 1988). These fungal toxins also produced by strains of some species of *Verticillium* (Nilanonta, Isaka et al. 2003) and *Halosarpheia* (Lin, Wang et al. 2002).

Enniatin biosynthesis is carried out by compounds like l-valine, l-leucine or l-isoleucine, d-2-hydroxy-3-methylbutanoic, d-2,3-dihydroxy-3-methylpentanoic and d-2-hydroxy-3-methylpentanoic acids, ATP, and S-adenosylmethionine (Firakova, Proksa et al. 2007). The structural variants of enniatin are enniatin A, A₁, B and B₁ with minor concentrations of enniatin C, D, E and F. The production of enniatins is catalysed by enniatin synthetase, which was purified from *Fusarium scirpi*, *F. oxysporum*, *F. lateritium* and *F. sambucinum* (Zocher, Keller et al. 1982, Pieper, Kleinkauf et al. 1992). Enniatin synthetase works as constitutive enzyme in primary metabolism and it does not regulated by transcriptional or a translational level (Billich and Zocher 1988).

Enniatins as an ionophore are very much interesting for membrane researchers as they have some common properties like cyclo-octadepsipeptide valinomycin (MacDonald 1969). Biochemically, enniatins are lipophilic ionophores which are incorporated into the cell membrane and forming cation selective pores and transmembrane spanning (Firakova, Proksa et al. 2007)

Enniatins are common contaminants for grain-based food and feed (Behm, Degen et al. 2009, Watjen, Debbab et al. 2009). *F. avenaceum* enniatins plays a role during infection process of plants as they contribute to the fungal virulence on potato tuber tissue (Herrmann, Zocher et al. 1996). Several studies shows that enniatins poses potent antimicrobial and antifungal abilities. Enniatin A, A1 and B1 showed moderate antimicrobial against *Candida albicans*, *Cryptococcus neoformans* and *Mycobacterium intracellulare* (Jayasinghe, Abbas et al. 2006). At non-toxic concentration, enniatins are proved to inhibit the effects of drug efflux pump, which cause multidrug resistance also in cancer cells (Firakova, Proksa et al. 2007). Enniatins have strong hypolipidemic activity as in HepG2 cell line enniatin B was shown to inhibit ACAT activity, triglyceride biosynthesis and diminished cellular free fatty acids pool (Trenin, Tolstykh et al. 2000).

Due to of their apoptotic effects, enniatins are listed together with strong anticancer agents of natural origin (Watjen, Debbab et al. 2009). Enniatins have cytotoxic capabilities (Hyun, Lee et al. 2009), potentially able to cause cell cycle arrest (Dornetshuber, Heffeter et al. 2007) and induce apoptosis in mammalian cells (Dornetshuber, Heffeter et al. 2007, Behm, Degen et al. 2009, Tonshin, Teplova et al. 2010). Mixture of enniatins O1, O2, O3 proved cytotoxic against cancer cell lines KB,BC-1 and NCI-185 (Supothina, Isaka et al. 2004). Enniatins were tested *in vivo* for their anti-HIV activity (McKee, Bokesch et al. 1997). Enniatins do not exert oxidative stress or cause DNA damage (Behm, Degen et al. 2009). Their cytotoxic effects are mainly due to the damage of mitochondrial transmembrane potential, uncoupled oxidative phosphorylation, swelling of mitochondria and decreasing of mitochondrial ability to sequester Ca^{2+} . (Dornetshuber, Heffeter et al. 2007). Enniatins are recognized as potentially selective K^{+} ionophores in cellular membranes (Tonshin, Teplova et al. 2010). The apoptotic death induced by enniatins is caspase-dependent and is inhibited by the pancaspase inhibitor zVAD (Hyun, Lee et al. 2009). Further, enniatin were shown to involve in the inhibition of extracellular regulated protein kinase (ERK) as well as in the down regulation of NF- κ B (Watjen, Debbab et al. 2009).

4.9.2. Ochratoxin A

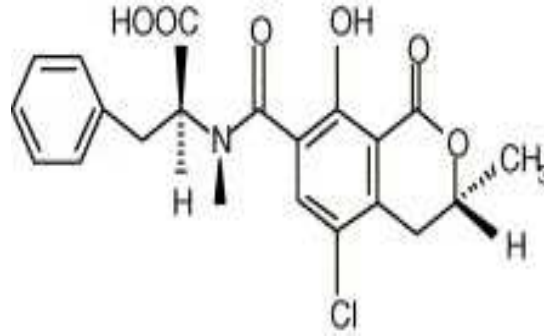


Figure 9. Chemical structure of ochratoxin A (Tafari, Ferracane et al. 2004).

Ochratoxin A (OTA) is a toxic fungal metabolite, produced by several subspecies of the genera *Penicillium* and *Aspergillus* (Zhang, Boesch-Saadatmandi et al. 2009). OTA found in foods with plant origin, edible animal tissues, human blood and tissues (Kuiper-Goodman and Scott 1989). Chemical structure in the figure 9 is showing that ochratoxin A is a pentaketide derived from the dihydrocoumarins family coupled to β -phenylalanine. Its chemical name is: L-phenylalanine -N- [(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyrane-7-yl) carbonyl] (R)isocoumarin. Several metabolites related to ochratoxin A have been discovered recently. Among all, its dechloro analog Ochratoxin B (OTB), its ethyl ester ochratoxin C (OTC), its isocoumaric derivative Ochratoxin α (Ota) and its dechloro analog, ochratoxin β (OT β) are famous.(el Khoury and Atoui 2010)

Ochratoxin A is known to have great health and agricultural significance. (Coronel, Sanchis et al. 2010, Duarte, Pena et al. 2011). In eastern Europe, the highest detected quantity of OTA in food of plant origin was about 5410 $\mu\text{g}/\text{kg}$ in rye flour of Poland and 3800 $\mu\text{g}/\text{kg}$ in barley of Czechoslovakia (Speijers and Vanegmond 1993). OTA proved very toxic to various animal species, the kidney being the main affected (Pohland, Nesheim et al. 1992). Some other effects related to OTA toxicity in animals includes cardiac and hepatic histological abnormalities, hemorrhage and thrombosis in the spleen, brain, liver, kidney and heart due to aberration of coagulation factors in rats (Albassam, Yong et al. 1987), myelotoxicity in mice (Boorman, Hong et al. 1984, Muller, Kielstein et al. 1995), gastro-intestinal tract and lymphoid tissues lesions in the hamster (Hagelberg, Hult et al. 1989), intestinal fragility and lesions of the kidney in chickens

(Elling, Hald et al. 1975), decreased feed intake in chickens after OTA exposure in feed (Prior, O'Neil et al. 1980). After mixing 2 ppm OTA in poultry feed, signs of ochratoxicosis including weight loss, low egg production, high water intake, diarrhea, excessive urination (Prior and Sisodia 1978) and hematological modifications (Bailey, Gibson et al. 1989) were noted. OTA has been known to inhibit protein synthesis in bacteria, yeast and mammalian cells in vitro and animals in vivo (Dirheimer and Creppy 1991).

As far as human health is concerned, ochratoxin A proved very toxic for organs such as kidney, liver, stomach, intestine, neurons, and immune cells (Zhang, Boesch-Saadatmandi et al. 2009, Duarte, Lino et al. 2011). Moreover, OTA has teratogenic potential (el Khoury and Atoui 2010). It is famous as a causative agent of Balkan endemic nephropathy and may contribute to the cause of urinary tract tumors (Mally and Dekant 2009, el Khoury and Atoui 2010). In renal epithelial cells, OTA activates the mitogen activated protein kinases ERK, JNK and p38. Activation of ERK results in mitosis, growth and differentiation, while JNK and p38 activation brings opposite effects (Sauvant, Holzinger et al. 2003). The nephrotoxicity of OTA may be due to mitochondrial dysfunction bringing to energy shortage and to the reactive oxygen species production. The treatment of isolated renal proximal tubules with OTA cause inhibition of mitochondrial respiration (Aleo, Wyatt et al. 1991). OTA also showed a powerful immunosuppressant effect at low or high doses as necroses of lymphoid tissues were observed after OTA treatment (Creppy, Betbeder et al. 1991). Humoral and cellular immunity were also compromised after OTA exposure (Holmberg, Thuvander et al. 1988). Further, OTA toxicity may cause iron deficiency anemia (Huff, Chang et al. 1979, Stoev, Anguelov et al. 2000).

Oxidative stress is a potential cause of toxicity and carcinogenicity induced by OTA as an elevated formation of the lipid peroxidation product malondialdehyde (MDA) was found in rats treated orally with 120 mg/kg bw/day of OTA for more than 60 days (Petrik, Zanic-Grubisic et al. 2003). Along with toxicity, OTA proved very effective as an inducer of suicidal death in many types of cell (Bouaziz, Sharaf El Dein et al. 2008, Ranaldi, Caprini et al. 2009, Yoon, Cong et al. 2009, Zhang, Boesch-Saadatmandi et al. 2009, Chopra, Link et al. 2010, Cui, Xing et al. 2010). Mechanisms involved in the triggering of suicidal death of cells by OTA include oxidative stress (El Golli Bennour, Rodriguez-Enfedaque et al. 2009, Yenilmez, Isikli et al. 2010), JNK and p38 pathways (Gekle, Sauvant et al. 2005, Sauvant, Holzinger et al. 2005) and Ca^{2+} entry into the cell (Klaric, Zeljezic et al. 2012).

4.9.3 Zearalenone

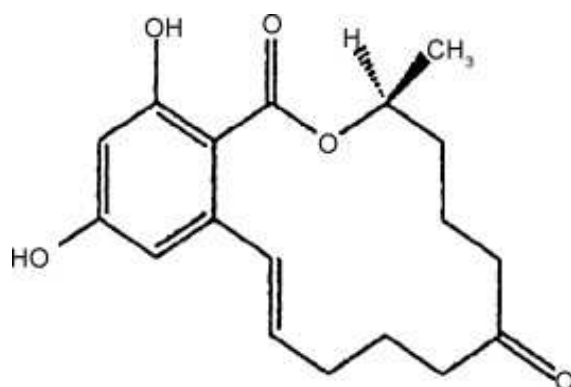


Figure 9. Chemical structure of zearalenone (Gromadzka, Waskiewicz et al. 2009).

Zearalenone (ZEA; previously named as F-2 toxin) is a non-steroidal oestrogenic mycotoxin produced by a variety of *Fusarium* fungi, including *F. graminearum* (*Gibberella zeae*), *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. crookwellense* and *F. semitectum*, which are common soil fungi and are worldwide famous cereal crops contaminants (Bennett and Klich 2003). Figure 9 depicts that, biochemically ZEA is a resorcyclic acid lactone (6-(10-hydroxy-6-oxo-trans-1-undecenyl)-B-resorcyclic acid lactone) (Zinedine, Soriano et al. 2007). The favorable conditions for ZEA production is high humidity and low temperature (Caldwell, Tuite et al. 1970). ZEA found as a very stable compound as it does not degraded at high temperature. (Kuiper-Goodman, Scott et al. 1987).

Fungi responsible for the production of ZEA and its metabolites mainly colonize in corn and to a lesser extent in barley, oats, wheat, sorghum, millet and rice. Additionally, ZEA also found in some cereal based products like flour, malt, soybeans and beer. (Zinedine, Soriano et al. 2007). ZEA derivatives like α -zearalenol, β -zearalenol, α -zearalanol, β -zearalanol and zearalanone found in *Fusarium* infected corn (Bottalico, Visconti et al. 1985) and in rice (Richardson, Hagler et al. 1985). In comparison, α -zearalenol proved three times more estrogenic than β -zearalenol (Hagler, Mirocha et al. 1979). Animals studies showed that zearalenone metabolites alpha-zearalenol (alpha-ZEA) and beta-zearalenol (beta-ZEA) are produced by the biotransformation and subsequently conjugated with glucuronic acid (Zinedine, Soriano et al. 2007) therefore, the dietary intake of ZEA from meat and products has less significance (Creppy 2002).

The toxicity caused by ZEA and its metabolites may be acute, sub acute or chronic and the outcomes are reproductive defects, developmental abnormalities, carcinogenicity, genotoxicity and immunotoxicity (Zinedine, Soriano et al. 2007). Zearalenone and its derivatives affect human and animal reproduction by interacting with estrogen receptors as was shown *in vitro*. (Yazar and Omurtag 2008). The reduced form of α -zearalenol found as an anabolic substance. In post-menopausal women, it is used for the hormonal replacement and as an oral contraceptive (Pazaiti, Kontos et al. 2012). ZEA is also capable to induce biphasic changes in the luteinizing hormone but not the follicle stimulating hormone (Bongiovanni 1983). ZEA exposure may stimulate pubertal development (Massart and Saggese 2010). Hematopoietic progenitor cells are also effectors of the toxicity of ZEA (Ficheux, Sibiril et al. 2012). Young children after exposure to zearalenone showed some precocious pubertal changes, which may be due to ZEA toxicity (Saenz de Rodriguez 1984). In bovine lymphocytes, ZEA causes genotoxicity by triggering DNA-adduct formation (Lioi, Santoro et al. 2004).

At lower concentrations, ZEA may have pro-apoptotic effect (Ahamed, Foster et al. 2001) but at higher concentrations ZEA is found as a strong apoptotic compound for different kind of cells (Abid-Essefi, Baudrimont et al. 2003, Kim, Son et al. 2003, Minervini, Giannoccaro et al. 2006, Doric, Radovic et al. 2007, Kouadio, Dano et al. 2007, Ayed-Boussema, Bouaziz et al. 2008, Banjerdpongchai, Kongtawelert et al. 2010, Malovrh and Jakovac-Strajn 2010, Salah-Abbes, Abbes et al. 2010, Yu, Zheng et al. 2011). Hyperestrogenism due to ZEA exposure triggers apoptosis and decreases the proliferative ability of follicular cells (Gajecka 2013). Signaling for the suicidal death induced by zearalenone includes CD95 (Jee, Noh et al. 2010), mitogen-activated protein kinases (Yu, Zheng et al. 2011), transcription factor p53 (Ayed-Boussema, Bouaziz et al. 2008, Yu, Zheng et al. 2011), Bax expression (Bouaziz, Martel et al. 2009, Banjerdpongchai, Kongtawelert et al. 2010), reactive oxygen species (Bouaziz, Martel et al. 2009, Banjerdpongchai, Kongtawelert et al. 2010, Salah-Abbes, Abbes et al. 2010, Yu, Zheng et al. 2011), mitochondrial depolarization (Ayed-Boussema, Bouaziz et al. 2008, Bouaziz, Sharaf El Dein et al. 2008, Banjerdpongchai, Kongtawelert et al. 2010, Zhu, Yuan et al. 2012), apoptosis-inducing factor (Yu, Zheng et al. 2011), endoplasmic reticulum stress (Banjerdpongchai, Kongtawelert et al. 2010), caspase activation (Zhu, Yuan et al. 2012) and increase in intracellular Ca^{2+} (Vlata, Porichis et al. 2006).

4.10. Phytochemicals

Phytochemicals are the chemical compounds which are isolated from plants by using highly sophisticated biochemical and analytical approaches. Plants are considered as a potent source of many drugs such as antispasmodics, emetics, anti-cancer, antimicrobials, and anticoagulants. Phytochemicals are non-nutritive constituents of the diet of plant origin, having anticarcinogenic and antimutagenic effects (Surh 2003). Some of the important examples of phytochemicals with pharmacological properties are vitamins, curcumin, capsaicin, gingerol, epigallocatechin-3-gallate, genistein, dulphoraphane, indole-3-carbinol, diallyl sulphide, caffeic acid phenethyl ester and resveratrol.

4.10.1. Withaferin A

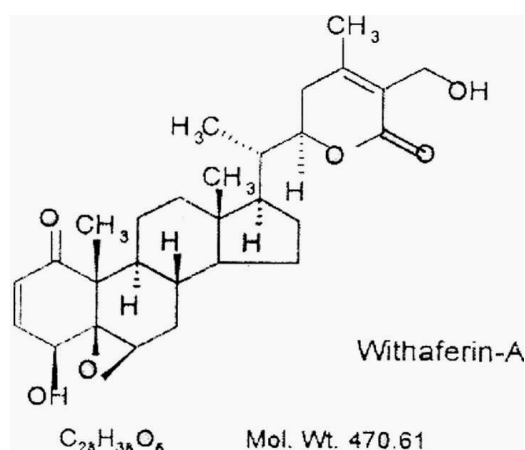


Figure 10. Chemical structure of Withaferin A (Debnath, Chattopadhyay et al. 2012).

Withania somnifera is an Indian medicinal plant, famous as *Indian ginseng* because of its beneficial effects on human health. *W. somnifera* found mainly in India, Pakistan, Afghanistan, Sri Lanka, Congo, South Africa, Egypt, Morocco, and Jordan (Dafni and Yaniv 1994, Kulkarni and Dhir 2008). It has anti-inflammatory, antitumor, antistress, antioxidant, immunomodulatory and hemopoietic effects. Also has beneficial role for the endocrine, cardiopulmonary, and central nervous systems (Mishra, Singh et al. 2000). Withanolides are biologically active compounds extracted from *W. somnifera*, including alkaloids, steroidal lactones, saponins containing an

additional acyl group, sitoindosides, flavonoids and tannins (Mishra, Singh et al. 2000, Kuo, Kuo et al. 2006, Bandyopadhyay, Jha et al. 2007)

Withaferin A is a triterpenoid Withanolide from plant *Withania somnifera* (Mirjalili, Fakhr-Tabatabaei et al. 2009). Figure 10 depicts withaferin A as 4 β , 27-dihydroxy-1-oxo-5 β , 6 β -epoxywitha-2-24-dienolide compound. It is a steroidal lactone which binds to vimentin and inhibits it (Bargagna-Mohan, Hamza et al. 2007).

Withaferin A structure gives insight about its cytotoxicity. Fuska and his co worker found that the unsaturated A ring of withaferin A was crucial for the cytotoxicity. Withaferin A derivatives with dissociated double bond possessed little cytotoxicity while the C27 hydroxyl group and the α , β -unsaturated δ -lactone ring with double bond at C24-25 were not essential for the cytotoxicity induced by withaferin A (Fuska, Fuskova et al. 1984). Further, 4 β -hydroxy-5 β , 6 β -epoxy-2-en-1-one moiety was crucial for the stimulation of cell differentiation by withaferin A but α , β -unsaturated δ -lactone ring was not important for same activity (Kuroyanagi, Shibata et al. 1999), similarly, 5 β , 6 β -epoxide group considered very important for withanolides cytotoxicity (Damu, Kuo et al. 2007).

Withaferin A is anti-inflammatory (Kaileh, Vanden Berghe et al. 2007, Sabina, Chandal et al. 2008, Maitra, Porter et al. 2009) may be due to NF κ B inhibition (Maitra, Porter et al. 2009), anti-tumor (Devi, Sharada et al. 1995, Sharada, Solomon et al. 1996), anti-angiogenesis (Mohan, Hammers et al. 2004), radio-sensitizing activity (Devi, Kamath et al. 2000), chemopreventive (Manoharan, Panjamurthy et al. 2008), and immunosuppressive (Shohat, Kirson et al. 1978). Withaferin A has anti tumor effect (Devi 1996, Mirjalili, Fakhr-Tabatabaei et al. 2009). Withaferin A may inhibit tumor growth by stimulating apoptosis of nucleated cells (Bargagna-Mohan, Hamza et al. 2007, Sen, Banerjee et al. 2007, Srinivasan, Ranga et al. 2007, Oh, Lee et al. 2008, Park, Rayalam et al. 2008, Samadi, Tong et al. 2010, Zhang, Samadi et al. 2012). Withaferin A induce apoptosis by several ways, which includes modification of gene expression (Stan, Hahm et al. 2008, Maitra, Porter et al. 2009, Grover, Shandilya et al. 2010, Koduru, Kumar et al. 2010, Lee, Hahm et al. 2010, Yu, Hamza et al. 2010, Hahm, Lee et al. 2011, Munagala, Kausar et al. 2011), mitochondrial depolarization (Malik, Kumar et al. 2007, Mandal, Dutta et al. 2008, Mayola, Gallerne et al. 2011, Yang, Choi et al. 2011), oxidative stress (Malik, Kumar et al. 2007, Hahm, Lee et al. 2011, Mayola, Gallerne et al. 2011, Yang, Choi et al. 2011), induction of endoplasmic reticulum stress (Choi, Park et al. 2011), Par-4 induction (Srinivasan, Ranga et al.

2007), p38 MAP kinase activation (Mandal, Dutta et al. 2008), inhibition of JAK/STAT3 (Um, Min et al. 2012) and FOXO3a- and Bim-dependent apoptosis (Stan, Hahm et al. 2008), inhibit protein kinase C (PKC) activity in leishmanial cells (Sen, Banerjee et al. 2007). The signal transduction and activation of transcription 3 (STAT3) may be molecular target of withaferin A (Lee, Hahm et al. 2010). Withaferin A showed *in vivo* anticancer activity in pancreatic cancer cells by inhibition of Hsp90 via an ATP-independent mechanism, cause Hsp90 client protein degradation (Yu, Hamza et al. 2010).

4.10.2. Oridonin

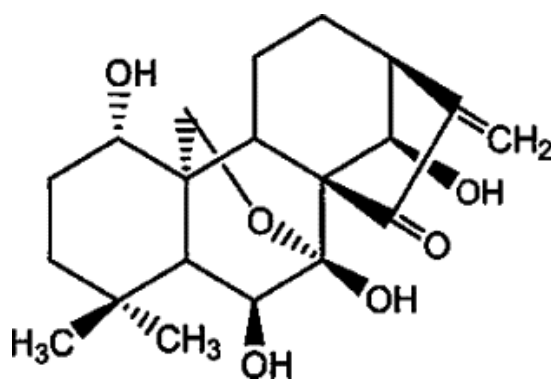


Figure 11. Chemical structure of oridonin (Lou, Gao et al. 2011)

Oridonin is an active tetracycline diterpenoid (Figure 11) compound, purified from traditional Chinese herb *Rabdosia rubescens* (Liu, Ouyang et al. 2012) alternatively known as rubesecensin A (Du, Villeneuve et al. 2008). Isodon plant *Rabdosia rubescens*, also famous as Donglingcao. The aerial parts of RR and other species from the same genus performed many functions including heat, toxicity, nourishment, blood stasis, and relief from swelling. *Rabdosia rubescens* has been used for the treatment of stomach ache, sore throat and cough. Moreover, RR and its extracts are capable to control disease progress, reduction in tumor mass and enhanced survival in patients suffering from gastric carcinoma, esophageal, liver and prostate cancer (Zhou, Chen et al. 2007).

Oridonin was identified for the first time in 1967 from *Rabdosia rubescens* and subsequently synthesized in 1973 (Li, Wang et al. 2011). As far as the stability is concerned, oridonin showed high stability in rabbit plasma, as both stock solutions and working solutions were over 99% of the nominal concentrations after storage at -20 °C for 30 days and 4 °C for 7 days (Mei, Xu et al. 2008). Additionally, oridonin in rat plasma retain its stability at room temperature for 12 h and -

20 °C for 30 days (Du, Liu et al. 2010). In medical applications, oridonin has some limitations as a therapeutic substance due to its lower solubility, therefore cytotoxicity of oridonin can be increased by adopting new ways for the preparation of oridonin nanosuspension and nanoparticle (Lou, Zhang et al. 2009). Production of derivative could be another possibility to enhance the cytotoxicity of oridonin. (Xu, Yang et al. 2008).

Oridonin exhibits potential anti-tumor capability (Gao, Zhang et al. 2008). Oridonin also showed therapeutic effectiveness as an immunosuppressive and anti-inflammatory (Hu, Du et al. 2008, Xu, Xue et al. 2009) and may be beneficial for the treatment of pulmonary hypertension (Wang, Sun et al. 2009). Treatment with cause modification in gene expression, induce oxidative stress, cell cycle arrest, senescence, autophagy, apoptosis and phagocytosis of apoptotic cells (Hsieh, Wijeratne et al. 2005, Ikezoe, Yang et al. 2005, Hu, Yang et al. 2007, Cheng, Qiu et al. 2009, Gao, Tang et al. 2010, Gao, Hu et al. 2010).

Effectiveness against several cancer cells, give identification to oridonin and its derivatives as potent anticancer agents (Zhou, Kang et al. 2007, Zhu, Xie et al. 2007, Lou, Zhang et al. 2009, Li, Wang et al. 2011). Both In vitro and in vivo studies showed that oridonin possesses anti-proliferative activity against breast, prostate, non-small cell lung cancer, glioblastoma multiforme (Zhang, Wu et al. 2004), human fibrosarcoma (HT1080 cells), human epidermoid carcinoma (A431 cells), murine aneuploid fibrosarcoma (L929 cells), human colon carcinoma HT29 cells, P388 lymphocytic leukaemia and Ehrlich ascites (Node, Sai et al. 1983, Li, Cui et al. 2007, Zhu, Xie et al. 2007, Cheng, Qiu et al. 2008, Zhang, Wu et al. 2009). Oridonin may be useful for the treatment of lymphoid malignancies, including adult T-cell leukemia and other types of cancers in which NF- κ B activation is involved. Similarly, oridonin inhibits NF- κ B activity in inflamed synovial cells from patients suffering from rheumatoid arthritis. (Ikezoe, Yang et al. 2005).

Along with autophagy and apoptosis, oridonin has been shown to stimulate G2/M phase cell cycle arrest in L929 cells (Cheng, Qiu et al. 2009). It also induce suicidal death and senescence in colorectal cancer SW1116 cells in vivo (Gao, Hu et al. 2010). Further, oridonin not only induced typical mitochondrial suicidal cell death in acute myeloid leukemic (AML) cells (Chen, Gao et al. 2005) but in murine models, also showed potential anti-leukemia effect with a low side-effect (Zhou, Chen et al. 2007).

Signaling for apoptosis triggers by oridonin includes, oxidative stress, calpain activity, DNA damage, histone (H3 and H4) hyperacetylation, p38 MAP kinase activation, p21, p27, p16, p53, cyclin B1/p-Cdc2, inhibition of tyrosine kinase activity, PI3 kinase pathway and NF κ B,

Ras/Raf/ERK signal pathway, suppression of c-myc expression, mitochondrial depolarization and cytochrome c release and caspase activation (Zhang, Wu et al. 2004, Hu, Yang et al. 2007, Li, Wu et al. 2008, Cheng, Qiu et al. 2009, Cheng, Qiu et al. 2009, Cho, Moon et al. 2009, Zhang, Wu et al. 2009, Feng, Zhang et al. 2011, Gatsinzi and Iverfeldt 2011). At a low sub toxic dosage, oridonin protects against arsenic-induced cytotoxicity by the activation of Nrf2 signaling pathway and was capable to stabilize Nrf2 by the inhibition of Nrf2 ubiquitination and degradation which lead to the accumulation of the Nrf2 protein and activation of the Nrf2-dependent cyto protective response. (Du, Villeneuve et al. 2008).

4.10.3. Dicoumarol

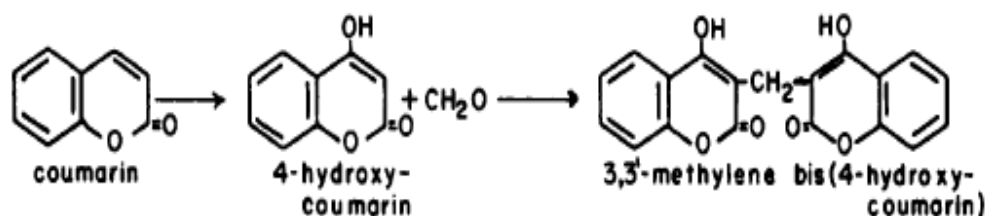


Figure 12. Biosynthesis of Dicoumarol (Link 1959)

Natural anticoagulant dicoumarol or 3,3'-methylenebis[4-hydroxycoumarin] (Figure 12) is a derivative of coumarin (Madari, Panda et al. 2003). It is metabolized from coumarin in the sweet clover like *Melilotus alba* and *Melilotus officinalis* by the action of like molds *Penicillium nigricans* and *Penicillium jensi*. Coumarin (1,2-benzopyrone) is the simple compound from a large class of naturally occurring phenolic substances made by fusion of the benzene and α -pyrone rings (Madari, Panda et al. 2003). Coumarin and its derivatives expressed various antitumor and antiproliferative effects. *In vitro* studies showed that the coumarin compounds stops proliferation of human malignant cell lines (Marshall, Mohler et al. 1994, Brar, Kennedy et al. 2001). Similarly, *in vivo* experiments expressed anti tumor activity (Feuer, Kellen et al. 1976, Maucher and von Angerer 1994, Madari, Panda et al. 2003). In clinical trials coumarin compounds proved anti proliferative against prostate cancer, malignant melanoma and metastatic renal cell carcinoma (Marshall, Butler et al. 1991, Mohler, Gomella et al. 1992, Thornes, Daly et al. 1994). Additionally, the coumarin anticoagulants, dicoumarol and its synthetic derivative

warfarin sodium proved capable to decrease metastases in animal models (Smith, Neubauer et al. 1988).

Dicoumarol is also famous as vitamin K antagonist (Stirling 1995, Lotrionte, Castagno et al. 2009). In humans, dicoumarol is slowly metabolized to unknown transformation products. The drug is mainly localized on the proteins of plasma and in other body tissues. Dicoumarol is slowly absorbed from the gastrointestinal tract and there is considerable variation in the absorption rate among different individuals. Furthermore, the small doses are absorbed quickly than large and similarly, the rate of transformation also depends on the dose as the smaller doses metabolized with a faster rate (Weiner, Shapiro et al. 1950).

Dicoumarol has multiple side effects (Nolan, Scott et al. 2010, Scott, Barnes et al. 2011). One of the side effects of dicoumarol treatment is anemia, may be due to excessive bleeding (Drucker-Colin, Jaques et al. 1971, Runciman, Lee et al. 2002). Dicoumarol proved very effective for the treatment of acute arterial occlusions (Petit and Berne 1947). Dicoumarol is not used only to increase the prothrombin time of patients but also to prevent thrombosis.(Jaques 1959). It may sensitize cells against the cytotoxicity of different chemicals (Watanabe, Nishiyama et al. 2006, Cabello, Bair et al. 2009). On the other hand, dicoumarol may accelerate cytotoxicity (Wondrak 2007, Dong, Bey et al. 2010). It is also proved hepatotoxic as it affects the plasma concentration of fibrinogen which is produced in the liver as well as proved toxic for the heart muscles and may be cause heart failure. (Jaques 1959). In the liver, dicoumarol presumably inhibits an enzyme network. (Martius and Nitz-Litzow 1955).

Cytotoxicity induced by dicoumarol is not always interfere with vitamin K epoxide oxidoreductase or a previously unidentified features of vitamin K metabolism, as the vitamin K addition does not effect the anti proliferation of cells by dicoumarol (Du, Daniels et al. 2006). Antiproliferative effects of dicoumarol possibly by stabilizing spindle microtubule dynamics through a unique interaction with tubulin and microtubules (Madari, Panda et al. 2003). Inhibition of cell growth by dicoumarol may be relatively specific for different tumor cells, since production of normal airway myocytes of human origin is not affected (Brar, Kennedy et al. 1999). Dicoumarol and other coumarin compounds are as used cancer therapy but mechanism of action of these compounds is not so much clear (Du, Daniels et al. 2006). In human pancreatic cancer cells dicoumarol triggers cytotoxicity and oxidative stress (Du, Daniels et al. 2006). Dicoumarol

induced oxidative stress by intracellular inhibition of NQO1, leads to increased cell toxicity (Hosoda, Nakamura et al. 1974, Hollander and Ernster 1975). Studies to find out the cytotoxic effects of dicoumarol demonstrate that the mitochondrial metabolism might be part of the mechanism (Winski, Koutalos et al. 2002, Lewis, Ough et al. 2005). Dicoumarol induces its toxicity on cells by interfering with mitochondrial function and stimulation of superoxide production (Bello, Gomez-Diaz et al. 2005, Gonzalez-Aragon, Ariza et al. 2007). In transformed fibroblast cell lines, dicoumarol decreased clonogenic survival in IMRSV-90 as compared to IMR-90 (Du, Daniels et al. 2006).

5. AIM OF THE STUDY

The prime objectives of the study were to determine the mechanisms and signaling involved in the regulation of suicidal erythrocyte death induced by xenobiotics. To rule out the effective dosages for the induction of suicidal death and cytotoxicity, erythrocytes were treated with different food borne mycotoxins and therapeutically important phytochemicals under their reported plasma level.

In the first section of the study, erythrocytes were treated *in vitro* with different molar concentration of food contaminating mycotoxins enniatin A, ochratoxin A and zearalenone. Experiments were performed to measure cytosolic calcium activity, phosphatidylserine exposure on cell surface, cell volume changes, and ceramide formation by FACS analysis. To find out additional signaling pathways and elaborate the previous findings, further investigations like cytosolic ATP levels, hemolysis measurement, adhesion studies and confocal microscopy were performed.

In the second section of the study, *in vitro* experiments were performed after treatment of erythrocytes with different molar concentration of phytochemicals withaferin A, oridonin and dicoumarol. Intracellular Ca^{2+} activity, phosphatidylserine exposure on cell surface, cell volume changes, ceramide formation and ROS formation were determined by FACS analysis. To find additional signaling mechanisms involved in eryptosis and strengthen our previous observations, further experiments like cytosolic ATP concentration, patch clamp recordings, hemolysis measurement and confocal microscopy were also performed.

6. MATERIAL AND METHODS

6.1. Stimulation of suicidal death of erythrocytes by mycotoxins

6.1.1. Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes (aged 1-2 weeks) were kindly provided by the blood bank of the University of Tübingen. Viability of erythrocytes may depend on the donor and the storage time thus causing some inter-individual variability. To avoid any bias potentially introduced by the use of different erythrocyte batches, comparison was always made within a given erythrocyte batch. The study was approved by the ethics committee of the University of Tübingen (184/2003V).

Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl₂; pH 7.4 at 37°C for 48 hours. Where indicated, extracellular glucose was removed. Erythrocytes were exposed to enniatin A (Enzo, Lörrach, Germany), ochratoxin A (Enzo, Lörrach, Germany) and Zearlenone (Enzo, Lörrach, Germany), added at the indicated concentrations. In Ca²⁺-free Ringer solution, 1 mM CaCl₂ was substituted by 1 mM glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). Where indicated, the caspase inhibitor zVAD (Enzo, Lörrach, Germany) was used at a concentration of 10 µM, the protein kinase C inhibitor staurosporine (Sigma, Freiburg, Germany) was used at a concentration of 500 nM and oxidative stress was induced utilizing 0.3 mM *tert*-butyl hydroperoxide [*t*-BOOH] (Sigma, Freiburg, Germany).

All incubations (48 hours) were carried out in Ringer solution containing 1 mM CaCl₂ or where indicated in Ca²⁺-free Ringer solution. Following pretreatment with the respective Ca²⁺ containing or Ca²⁺ free solutions for the indicated time periods the erythrocytes were washed with Ringer solution containing 5 mM CaCl₂ and resuspended for 20 minutes with the same solution containing Annexin V-Fluos. The time period of 20 minutes is considered too short for the triggering of eryptosis.

6.1.2. FACS analysis of annexin V-binding and forward scatter

After incubation under the respective experimental conditions, 50 µl cell suspension were washed in Ringer solution containing 5 mM CaCl₂ and then stained for 20 minutes with Annexin-V-Fluos (1:500 dilution; Roche, Mannheim, Germany) under protection from light [32]. In the following, the

forward scatter (FSC) of the cells was determined and annexin V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS calibur (BD, Heidelberg, Germany).

6.1.3. Measurement of intracellular Ca^{2+}

After incubation 50 μl suspension erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM CaCl_2 and 2 μM Fluo-3/AM. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM CaCl_2 . The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer. Then, Ca^{2+} -dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

6.1.4. Determination of ceramide formation

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation with and without ochratoxin A, cells were stained for 1 h 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 1:5. The samples were washed twice with PBS-BSA. Subsequently, 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 then analyzed by flow cytometric analysis in FL-1.

6.1.5. Determination of intracellular ATP concentration

For determination of intracellular ATP, 90 μl of erythrocyte pellets were incubated for 48 h at 37°C in Ringer solution with or without enniatin A (final hematocrit 5%). Additionally, erythrocytes were also incubated in glucose depleted Ringer solution as a positive control. All manipulations were then performed at 4°C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of HClO_4 (5%). After centrifugation, an aliquot of the supernatant (400 μl) was adjusted to pH 7.7 by addition of saturated KHCO_3 solution. After dilution of the supernatant, the ATP concentrations of the aliquots were determined utilizing the luciferin–luciferase assay kit (Roche Diagnostics) on a luminometer (Berthold Biolumat LB9500, Bad Wildbad, Germany)

according to the manufacturer's protocol. ATP concentrations are expressed in mmol/l cytosol of erythrocytes.

6.1.6. Measurement of hemolysis

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

6.1.7. Cell culture and in vitro adhesion studies

Human umbilical vein endothelial cells (HUVEC) from early passage of culture were cultured in complete endothelial cell basal medium (PAA) containing growth factors and 10% fetal bovine serum and grown to confluency. HUVEC (5×10^5) were attached on sterile coverslips coated with 0.2% gelatine (Sigma-Aldrich) by overnight incubation in complete endothelial cell basal medium under cell culture conditions. Erythrocytes prepared as indicated were perfused on a HUVEC monolayer in a flow chamber model (Oligene) at arterial shear rates ($1,200^{-5}$). The interaction events were recorded with a charge-coupled device camera (Carl Zeiss) with $\times 20$ magnification, followed by analysis of the number of adherent erythrocytes per high powerfield.

6.1.8. Confocal microscopy and immunofluorescence

For the visualisation of eryptotic erythrocytes, 4 μ l of erythrocytes, incubated in respective experimental conditions, were stained with FITC-conjugated Annexin-V-Fluos (1:250 dilution; Roche, Mannheim, Germany) in 200 μ l Ringer solution containing 5 mM CaCl_2 . Then the erythrocytes were washed twice and finally resuspended in 50 μ l of Ringer solution containing 5 mM CaCl_2 . 20 μ l were smeared onto a glass slide, covered with a coverslip and images were subsequently taken on a Zeiss LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss MicroImaging, Oberkochen, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

6.1.9. Statistics

Data are expressed as arithmetic means \pm SEM. Statistical analysis was made using paired ANOVA with Tukey's test as post-test, as appropriate. n denotes the number of different erythrocyte

specimens studied. The batches of erythrocytes differed moderately in their susceptibility to eryptosis. Thus, the control values were not identical in all series of experiments. To avoid any bias potentially introduced by the use of different erythrocyte batches, comparison was always made within a given erythrocyte batch.

6.2 Stimulation of suicidal death of erythrocytes by phytochemicals

6.2.1. Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003V). Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl₂; pH 7.4 at 37°C for 48 h. Where indicated, erythrocytes were exposed to withaferin A (Enzo, Lörrach, Germany), oridonin (Enzo, Lörrach, Germany), dicoumarol (Sigma, Freiburg, Germany), amiloride and N-acetyl-L-cystein (both from Sigma, Freiburg, Germany) at the indicated concentrations. In Ca²⁺-free Ringer solution, 1 mM CaCl₂ was substituted by 1 mM glycol-bis-(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). Where indicated erythrocytes were incubated in the absence of MgSO₄ and in presence of 1 mM CaCl₂ and 1 mM EGTA.

All incubations (48 hours) were carried out in Ringer solution containing 1 mM CaCl₂ or where indicated in Ca²⁺-free Ringer solution. Following pretreatment with the respective Ca²⁺ containing or Ca²⁺ free solutions for the indicated time periods the erythrocytes were washed with Ringer solution containing 5 mM CaCl₂ and resuspended for 20 minutes with the same solution containing Annexin V-Fluos. The time period of 20 minutes is considered too short for the triggering of eryptosis.

6.2.2. FACS analysis of annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 50 µl 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. In the following, the forward scatter (FSC) of the cells was determined, and annexin-V fluorescence

intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

6.2.3. Measurement of intracellular Ca²⁺

After incubation erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl₂ and 2 μM Fluo-3/AM. The cells were incubated at 37°C for 30 min and washed twice in Ringer solution containing 5 mM CaCl₂. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer. Then, Ca²⁺-dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

6.2.4. Determination of ceramide formation

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation with and without withaferin A, cells were stained for 1 h at 37°C with 1 μg/ml anti-ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:5. The samples were washed twice with PBS-BSA. Subsequently, the cells were stained for 30 min 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 then analyzed by flow cytometric analysis in FL-1.

6.2.5. Determination of intracellular ATP concentration

For determination of intracellular erythrocyte ATP, 90 μl of erythrocyte pellets were incubated for 48 h at 37°C in Ringer solution with or without oridonin (final hematocrit 5%). Additionally, erythrocytes were also incubated in glucose depleted Ringer solution as a positive control. All manipulations were then performed at 4°C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of HClO₄ (5%). After centrifugation, an aliquot of the supernatant (400 μl) was adjusted to pH 7.7 by addition of saturated KHCO₃ solution. After dilution of the supernatant, the ATP concentrations of the aliquots were determined utilizing the luciferin–luciferase assay kit (Roche Diagnostics) on a luminometer (Berthold Biolumat LB9500, Bad Wildbad, Germany) according to the manufacturer's protocol. ATP concentrations are expressed in mmol/l cytosol of erythrocytes.

6.2.6. Measurement of hemolysis

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

6.2.7. Estimation of ROS formation

Oxidative stress was determined utilizing 2',7' dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 50 μ l suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA (Sigma, Schnellendorf, Germany) in Ringer solution containing DCFDA at a final concentration of 10 μ M. Erythrocytes were incubated at 37°C for 30 min in the dark and then washed three times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 μ l Ringer solution, and ROS-dependent fluorescence intensity was measured in the fluorescence channel FL-1 of a FACS calibur (BD).

6.2.8. Patch clamp recordings

Patch clamp recordings were performed at room temperature. The patch electrodes were made of borosilicate glass capillaries (150 TF-10, Clark Medical Instruments) using a horizontal DMZ puller (Zeitz). Pipettes with high resistance from 17 to 20 M Ω were connected via an Ag-AgCl wire to the headstage of an EPC 9 patch-clamp amplifier (HEKA). Data acquisition and data analysis were controlled by a computer equipped with an ITC 16 interface (Instrutech) and by using Pulse software (HEKA). For current measurements, erythrocytes were held at a holding potential (V_h) of -10 mV, 200 ms pulses from -100 to +100 mV were applied in increments of +20 mV. The original current traces are depicted without filtering (acquisition frequency of 3 kHz). The currents were analyzed by averaging the current values measured between 90 and 190 ms of each square pulse (current- voltage relationship). The applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. The offset potentials between both electrodes were zeroed before sealing. The liquid junction potentials between bath and pipette solutions, and between the bath solutions and the salt bridge (filled with NaCl bath solution) were calculated according to Barry and Lynch [40]. Data were corrected for liquid junction potentials. For whole cell recording, the pipette solutions consisted of (in mM): 125 Na-gluconate, 10 NaCl, 1 MgCl₂, 1 MgATP, 1 EGTA, 10 HEPES/NaOH (pH 7.4).

The NaCl Ringer's bath solution contained (in mM): 145 NaCl, 5 KCl, 2 MgCl₂, 1 CaCl₂, 5 glucose, 10 HEPES/NaOH (pH 7.4). Cl⁻ free bath solution contained (in mM): 150 Na-gluconate, 10 HEPES/NaOH (pH 7.4). The NMDG-Cl and CaCl₂ bath solutions contained (in mM): 180 NMDG or 100 CaCl₂, 10 HEPES (titrated with NMDG or CaOH₂ to pH 7.4). Chemicals were obtained from Sigma (Taufkirchen, Germany) and were of the highest grade available. Experiments were performed with or without a 3-6 hours exposure to dicoumarol. Sustained exposure resulted in eryptosis, which rendered the performance of patch clamp recordings difficult.

6.2.9. Confocal microscopy and immunofluorescence

For the visualisation of eryptotic erythrocytes, 4 µl of erythrocytes, incubated in the respective experimental conditions, were stained with FITC-conjugated Annexin-V-Fluos (1:250 dilution; Roche, Mannheim, Germany) in 200 µl Ringer solution containing 5 mM CaCl₂. Then the erythrocytes were washed twice and finally re-suspended in 50 µl of Ringer solution containing 5 mM CaCl₂. 20 µl were smeared onto a glass slide, covered with a coverslip and images were subsequently taken on a Zeiss LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss MicroImaging, Oberkochen, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

6.2.10. Statistics

Data are expressed as arithmetic means ± SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and paired *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.

7. RESULTS

7.1. Stimulation of suicidal death of erythrocytes by mycotoxins

7.1.1. Enniatin A

In a first series of experiments, Fluo 3 fluorescence was employed to estimate cytosolic Ca^{2+} concentration. As illustrated in Fig. 13A and 13B, exposure of erythrocytes for 48 hours to Ringer solution containing enniatin A was followed by an increase of cytosolic Ca^{2+} concentration. When compared to erythrocytes exposed for 48 hours to Ringer solution without enniatin A, the cytosolic Ca^{2+} concentration was significantly higher at $\geq 2.5 \mu\text{M}$ enniatin A concentration. Additional experiments explored the effect of enniatin A during glucose depletion. As illustrated in Fig. 13C and 13D, glucose withdrawal similarly increased cytosolic Ca^{2+} activity, an effect augmented in the presence of enniatin A. The difference in cytosolic Ca^{2+} activity of glucose depleted erythrocytes between the presence and absence of enniatin A reached statistical significance at $\geq 2.5 \mu\text{M}$ enniatin A concentration.

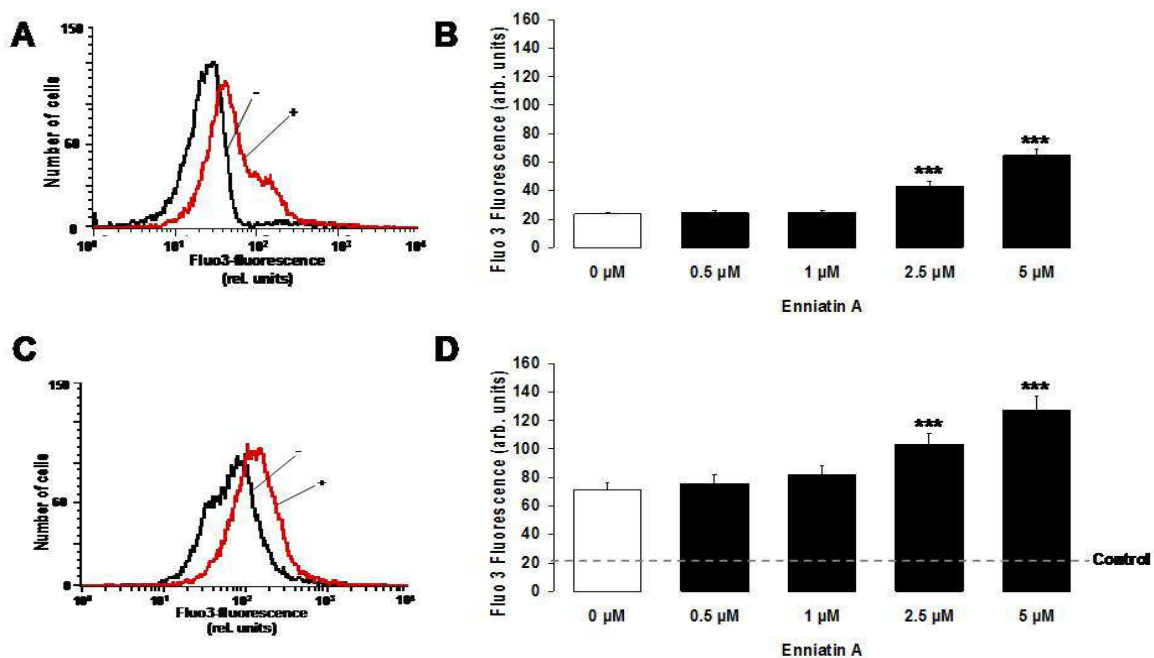


Fig. 13: Effect of enniatin A on erythrocyte cytosolic Ca^{2+} concentration

A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) the presence of 5 μM enniatin A. **B.** Arithmetic means \pm SEM ($n = 12$) of the geo means (geometric mean of the histogram in arbitrary units) of Fluo3 fluorescence in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) enniatin A. *** indicates significant difference ($p < 0.001$) from the respective value in the absence of enniatin A (ANOVA). **C.** Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without glucose, without (-, black line) and with the (+, red line) presence of 5 μM enniatin A. **D.** Arithmetic means \pm SEM ($n = 12$) of the geo means (geometric mean of the histogram in arbitrary units) of Fluo3 fluorescence in erythrocytes exposed for 48 hours to Ringer solution without glucose in absence (white bar) or in presence (black bars) of enniatin A. The broken line indicates control Fluo3 fluorescence in the presence of glucose and absence of enniatin A. *** indicates significant difference ($p < 0.001$) from the respective value in the absence of enniatin A (ANOVA).

As Ca^{2+} activates K^+ channels with subsequent exit of KCl and cell shrinkage, forward scatter was determined to reveal a possible effect of enniatin A on cell volume. As shown in Fig. 14A and 14B, exposure of erythrocytes for 48 hours to Ringer solution containing enniatin A was indeed followed by a decrease of forward scatter, an effect reaching statistical significance at 1 μM enniatin A. As shown in Fig. 14C and 14D, glucose withdrawal was again followed by a decrease of forward scatter, reflecting cell shrinkage. The administration of 1 μM enniatin A was followed by a further significant decrease of forward scatter. However, at a concentration of 5 μM enniatin A, the forward scatter of the glucose depleted erythrocytes was significantly higher than the forward scatter of glucose depleted erythrocytes in the absence of enniatin A.

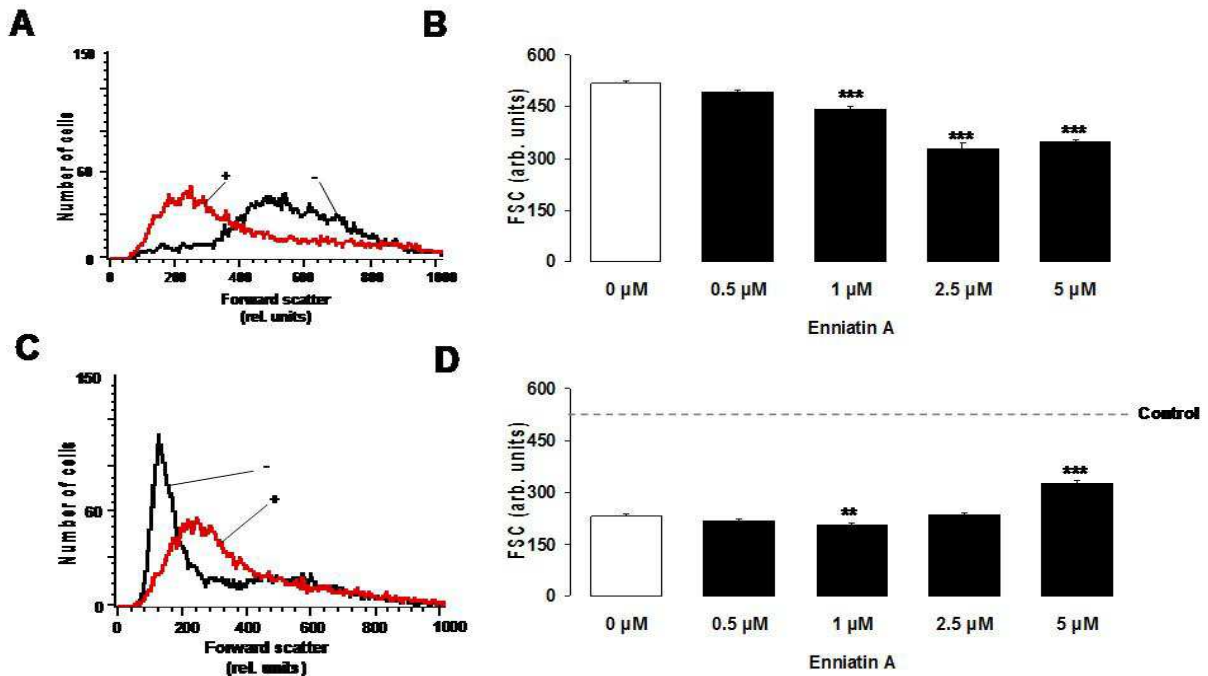


Fig. 14: Effect of enniatin A on erythrocyte forward scatter

A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) the presence of 5 μM enniatin A. **B.** Arithmetic means \pm SEM ($n = 12$) of the erythrocyte forward scatter following incubation for 48 hours in Ringer solution without (white bar) or with (black bars) 1-5 μM enniatin A. *** ($p < 0.001$) indicate significant difference from the absence of enniatin A (ANOVA). **C.** Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without glucose, without (-, black line) and with (+, red line) presence of 5 μM enniatin A. **D.** Arithmetic means \pm SEM ($n = 12$) of the erythrocyte forward scatter following incubation for 48 hours in Ringer solution without glucose, in the absence (white bar) or presence (black bars) of 1-5 μM enniatin A. The broken line indicates control forward scatter in the presence of glucose and absence of enniatin A. **, *** ($p < 0.01$, $p < 0.001$) indicate significant difference from the absence of enniatin A (ANOVA).

Ca^{2+} further stimulates cell membrane scrambling with phosphatidylserine exposure at the cell surface. To estimate an effect of enniatin A on cell membrane scrambling, phosphatidylserine exposing erythrocytes were identified utilizing annexin V-binding. In a first approach, annexin V binding cells were visualized utilizing confocal microscopy (Fig. 15). The number of annexin V positive erythrocytes was higher following treatment (for 48 hours) with enniatin A than following incubation (for 48 hours) in Ringer solution without enniatin A.

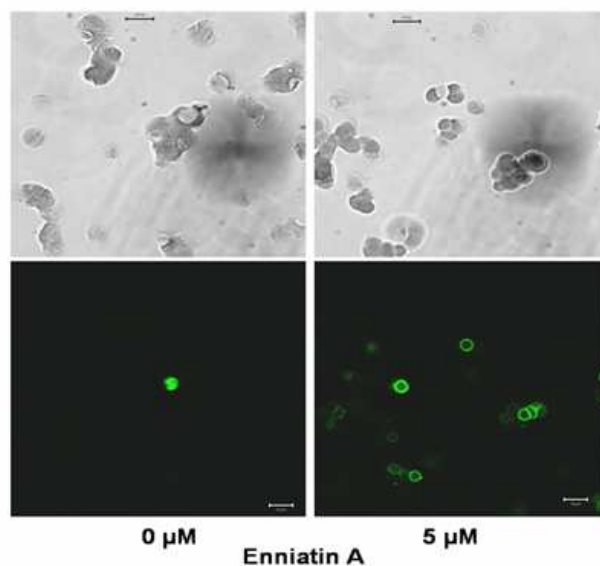


Fig. 15: Confocal images of PS-exposing erythrocytes exposed to enniatin A

Confocal microscopy of FITC-dependent fluorescence (lower panels) and light microscopy (upper panels) of human erythrocytes stained with FITC-conjugated Annexin-V-Fluos following 48 hours incubation in Ringer solution without (left panels) and with (right panels) 5 μM enniatin A.

To quantify the effect of enniatin A on cell membrane scrambling, annexin V-binding erythrocytes were counted in FACS analysis. As displayed in Fig. 16A and 16B, the percentage of annexin V binding erythrocytes was significantly higher following exposure of erythrocytes for

48 hours to Ringer solution containing $\geq 2.5 \mu\text{M}$ enniatin A than following exposure of erythrocytes for 48 hours to Ringer solution without enniatin A. Glucose withdrawal further enhanced the percentage of annexin V binding erythrocytes. As shown in Fig. 16C and 16D, the percentage of annexin V binding erythrocytes was higher in glucose depleted cells exposed to enniatin A than in glucose depleted cells without enniatin A exposure. The difference of the percentage annexin V binding erythrocytes between the presence and absence of enniatin A was statistically significant at $\geq 1 \mu\text{M}$ enniatin A.

Further experiments were performed to reveal a possible hemolytic action of enniatin A. To this end, hemoglobin release was determined following exposure of the erythrocytes to Ringer solution for 48 hours without or with 1-5 μM enniatin A. As indicated in Fig. 4B, the exposure to enniatin was followed by hemolysis, an effect reaching statistical significance at 2.5 μM enniatin A. Following enniatin A exposure, the percentage of hemolysed erythrocytes was, however, one magnitude smaller than the percentage of phosphatidylserine exposing erythrocytes. Collectively, the above results reveal that enniatin A triggers phospholipid scrambling and to a clearly lesser extent hemolysis.

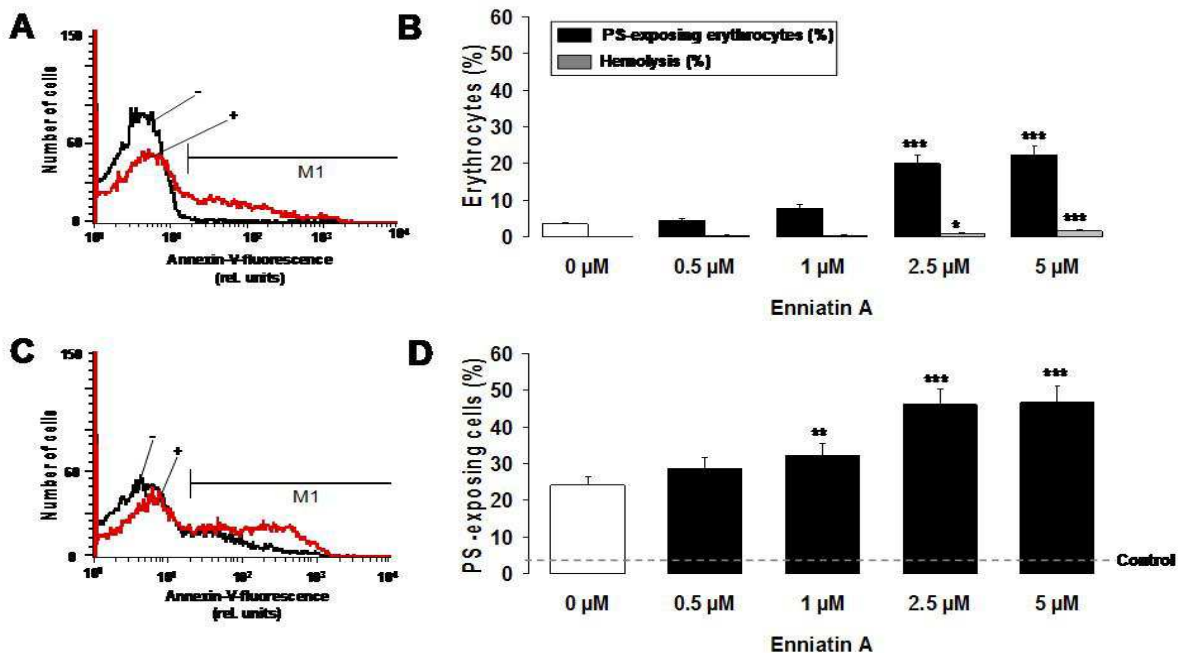


Fig. 16: Effect of enniatin A on phosphatidylserine exposure and erythrocyte membrane integrity

A. Original histogram of annexin V binding of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of 5 μM enniatin. **B.** Arithmetic means \pm SEM ($n = 12$) of erythrocyte annexin V binding following incubation for 48 hours in Ringer solution without (white bar) or with (black bars) the presence of 1-5 μM enniatin A. For comparison, arithmetic means \pm SEM ($n = 4$) of the percentage of hemolysis is shown as grey bars. *, *** ($p < 0.05$, $p < 0.001$) indicate significant difference from the absence of enniatin A (ANOVA). **C.** Original histogram of annexin V binding of erythrocytes following exposure for 48 hours to Ringer solution without glucose in the absence (-, black line) and presence (+, red line) of 5 μM enniatin A. **D.** Arithmetic means \pm SEM ($n = 12$) of erythrocyte annexin V binding following incubation for 48 hours in the absence of glucose in the absence (white bar) or presence (black bars) of 1-5 μM enniatin A. The broken line indicates control annexin V binding in the presence of glucose and absence of enniatin A. **, *** ($p < 0.01$, $p < 0.001$) indicate significant difference from the absence of enniatin A (ANOVA).

The parallel alteration of cytosolic Ca^{2+} activity and cell membrane scrambling was suggestive for a causal role of Ca^{2+} in the triggering of cell membrane scrambling following enniatin A exposure. To test, whether enniatin A-induced cell membrane scrambling was indeed dependent on Ca^{2+} , erythrocytes were treated with enniatin A in the presence and nominal absence of extracellular Ca^{2+} . As shown in Fig. 17A, the effect of enniatin A on annexin V-binding was significantly blunted but not fully abolished in the nominal absence of Ca^{2+} . Furthermore, the cation channel inhibitor amiloride (1 mM) blunted but not abolished the effect of enniatin A on annexin V-binding (Fig. 17B). Further experiments were performed to test, whether enniatin A-induced cell membrane scrambling required the activation of caspases. To this end, erythrocytes were treated with enniatin A in the absence and presence of the pancaspase inhibitor zVAD. As shown in Fig. 17D, the effect of enniatin A on annexin V-binding was not significantly modified by zVAD (10 μM). As a positive control, erythrocytes were treated with zVAD (10 μM) for 48 hours and then exposed to oxidative stress using *t*-BOOH (0.3 mM) for 30 minutes. zVAD treatment significantly attenuated the enhanced annexin V-binding induced by oxidative stress (Fig. 17E). Further experiments explored the impact of kinases on enniatin A induced cell membrane scrambling. The kinase inhibitor staurosporine (500 nM) blunted the effect of enniatin A on annexin V-binding (Fig. 17C).

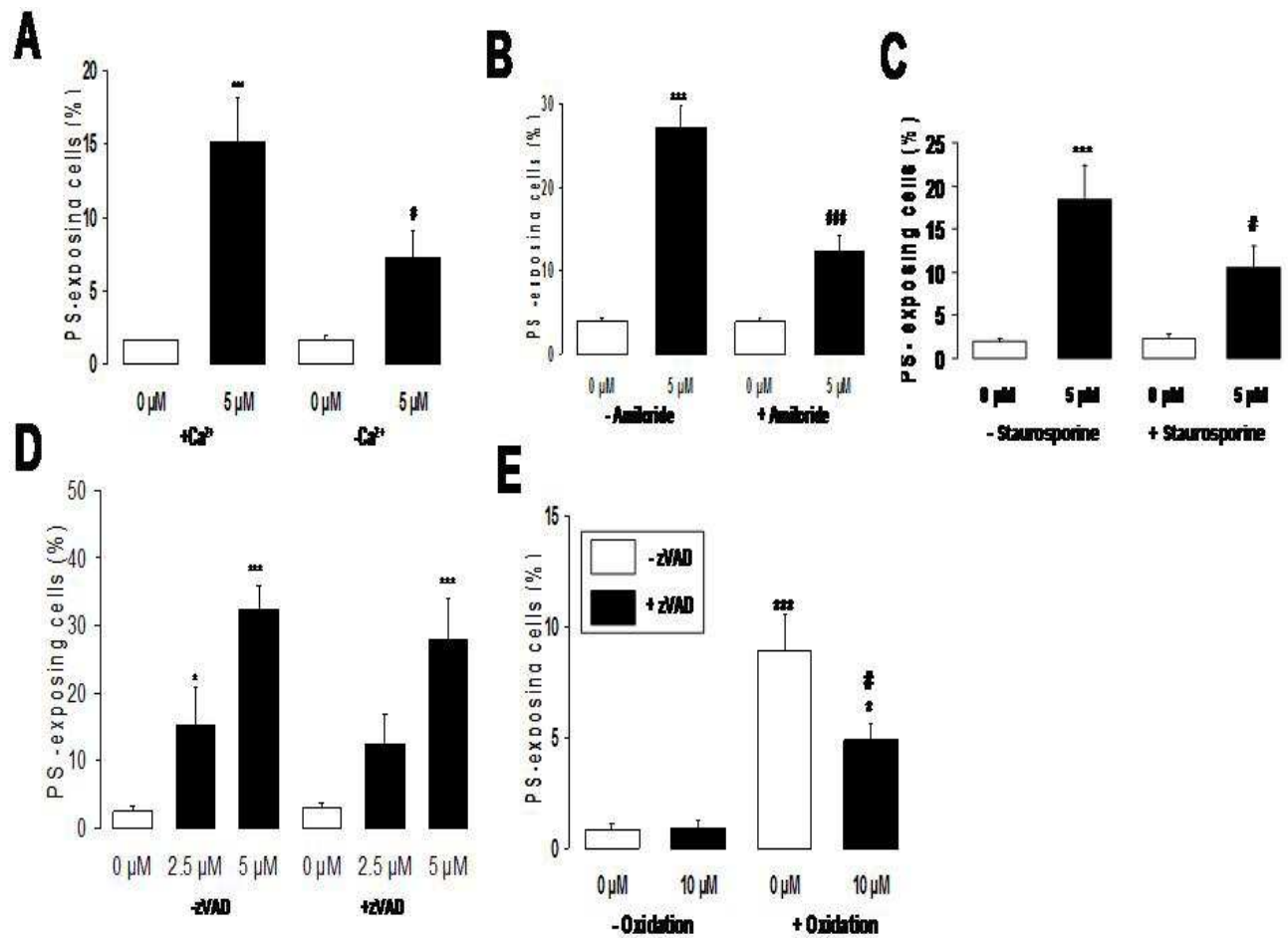


Fig. 17: Effect of Ca²⁺ withdrawal, cation channel, caspase and protein kinase C inhibition on enniatin A induced phosphatidylserine exposure

A. Arithmetic means \pm SEM (n = 4) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bar) or with (black bars) 5 μM enniatin A in the presence (left bars, +Ca²⁺) and absence (right bars, -Ca²⁺) of calcium. *** (p<0.001) indicate significant difference from the absence of enniatin A (ANOVA), # indicates significant difference (p<0.05) from the respective values in the presence of Ca²⁺. **B.** Arithmetic means \pm SEM (n = 5) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bar) or with (black bars) 5 μM enniatin A in the absence of amiloride (1 mM) (left bars) and presence of amiloride (1 mM) (right bars). *** (p<0.001) indicate significant difference from the absence of enniatin A (ANOVA), ### indicates significant difference (p<0.001) from the respective values in the absence of amiloride. **C.** Arithmetic means \pm SEM (n = 4) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bar) or with (black bars) 5 μM enniatin A in the absence (left bars, - staurosporine) and presence (right bars, + staurosporine) of staurosporine (500 nM). *** (p<0.001) indicate significant difference from the absence of enniatin A (ANOVA). # indicates significant difference (p<0.05) from the respective value in the absence of staurosporine. **D.** Arithmetic means \pm SEM (n = 4) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bar) or with (black bars) 2.5 μM and 5 μM enniatin A in the absence (left bars, - zVAD) and presence (right bars, +zVAD) of zVAD (10 μM). *, *** (p<0.05, p<0.001) indicate significant difference from the absence of enniatin A (ANOVA). **E.** Arithmetic means \pm SEM (n = 9) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bar) or with (black bars) 10 μM zVAD in the absence (left bars, - Oxidation) and presence (right bars,

+ Oxidation) of *t*-BOOH (0.3 mM). *, *** ($p < 0.05$, $p < 0.001$) indicate significant difference from the absence of *t*-BOOH (ANOVA). # indicates significant difference ($p < 0.05$) from the absence of zVAD.

Phospholipid scrambling could be triggered by energy depletion. Accordingly, additional experiments were performed to determine, whether enniatin A exposure influences intracellular ATP concentrations in erythrocytes. As shown in Fig. 18, a 48 hours exposure of human erythrocytes to enniatin A ($\geq 2.5 \mu\text{M}$) significantly reduced the cellular ATP concentration. Intracellular ATP concentration in erythrocytes incubated in glucose-depleted Ringer solution was similarly decreased significantly.

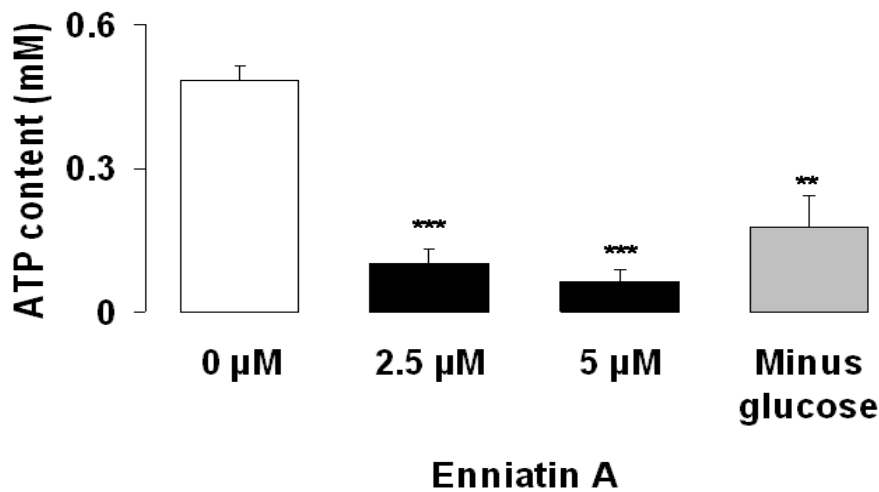


Fig. 18: *Effect of enniatin A on erythrocyte cytosolic ATP content*

Arithmetic means \pm SEM ($n = 4$) of the ATP concentration after a 48 hours incubation in Ringer solution without (white bar) or with (black bars) enniatin A at the indicated concentrations, or in glucose-depleted Ringer solution (grey bar, minus glucose) **, *** ($p < 0.01$, $p < 0.001$) indicates significant difference from control (absence of enniatin A and presence of glucose) (ANOVA).

7.1.2. Ochratoxin A

In order to determine, whether ochratoxin A stimulates Ca^{2+} -entry into erythrocytes, cytosolic Ca^{2+} -activity was estimated from Fluo3-fluorescence. Erythrocytes were loaded with Fluo3-AM and the Fluo3-fluorescence determined in FACS analysis. As illustrated in Fig. 19, a 48 hours exposure of human erythrocytes to ochratoxin A was followed by an increase of Fluo3-fluorescence, an effect reaching statistical significance at 2.5 μM ochratoxin A concentration.

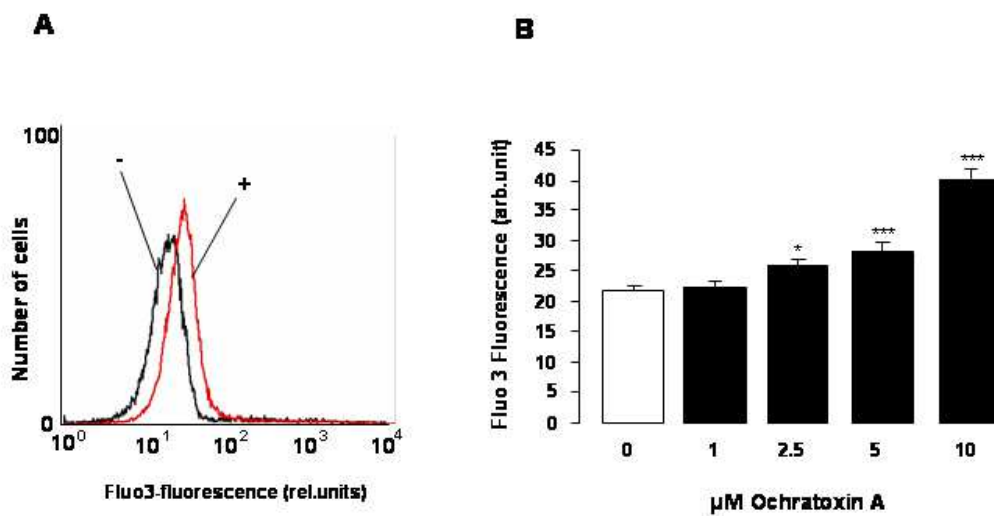


Fig. 19. Effect of ochratoxin A on erythrocyte cytosolic Ca^{2+} concentration.

A. Original histogram of Fluo3-fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of ochratoxin A (10 μM). **B.** Arithmetic means \pm SEM ($n = 12$) of the normalized geo means (geometric mean of the histogram in arbitrary units) of Fluo3-fluorescence in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) ochratoxin A (1-10 μM). * ($p < 0.05$), *** ($p < 0.001$) indicates significant difference from the absence of ochratoxin A (ANOVA).

An increased cytosolic Ca^{2+} -concentration is expected to stimulate Ca^{2+} -sensitive K^{+} -channels with subsequent K^{+} -exit, hyperpolarisation, Cl^{-} -exit and loss of cell water due to cellular loss of KCl . To explore, whether ochratoxin A influences cell volume, forward scatter was determined in FACS analysis. As shown in Fig. 20, a 48 h treatment with ochratoxin A was followed by a decrease of forward scatter, an effect reaching statistical significance at 5 μM ochratoxin A concentration.

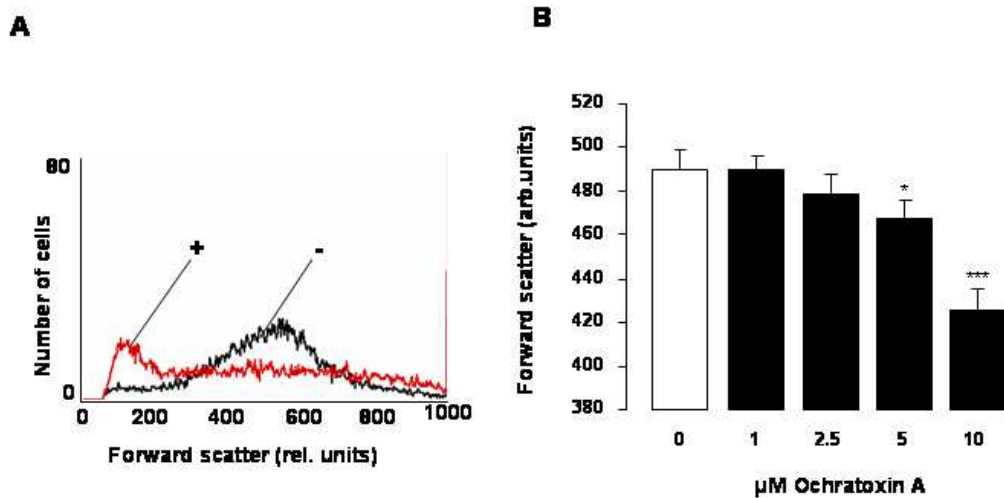


Fig. 20. Effect of ochratoxin A on erythrocyte forward scatter.

A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of ochratoxin A (10 µM). **B.** Arithmetic means ± SEM (n = 12) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) ochratoxin A (1-10 µM). * (p<0.05), *** (p<0.001) indicates significant difference from the absence of ochratoxin A (ANOVA).

Increased cytosolic Ca²⁺ is further expected to stimulate erythrocyte membrane scrambling with phosphatidylserine exposure at the erythrocyte surface. Accordingly, a further series of experiments explored whether ochratoxin A triggers cell membrane scrambling. Phosphatidylserine exposing erythrocytes were identified with binding of fluorescent annexin-V to the erythrocyte surface. As illustrated in Fig. 21, a 48 hours exposure to ochratoxin A resulted in an increase of annexin-V-binding, an effect reaching statistical significance at 2.5 µM ochratoxin A.

Further experiments addressed, whether ochratoxin A treatment triggers hemolysis, which was estimated from hemoglobin release into the supernatant. As shown in Fig. 21, exposure of erythrocytes to ochratoxin A for 48 hours led to a slight increase of hemoglobin concentration in

the supernatant, an effect reaching statistical significance at 10 μM .

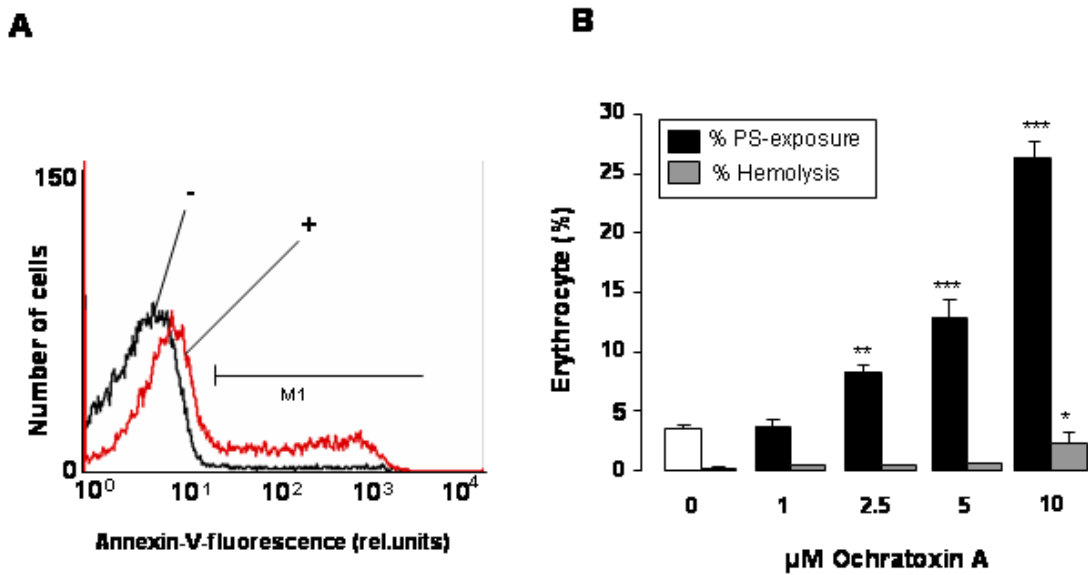


Fig. 21. Effect of ochratoxin A on phosphatidylserine exposure and hemolysis.

A. Original histogram of annexin V binding of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of ochratoxin A (10 μM). **B.** Arithmetic means \pm SEM of erythrocyte annexin-V-binding ($n = 12$) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of ochratoxin A (1-10 μM). For comparison, arithmetic means \pm SEM ($n = 4$) of the percentage of hemolysis is shown as grey bars. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) indicates significant difference from the absence of ochratoxin A (ANOVA).

An additional series of experiments explored, whether the ochratoxin A induced cell membrane scrambling resulted from Ca^{2+} entry. To this end, erythrocytes were treated with 10 μM ochratoxin A in the presence or in the nominal absence of extracellular Ca^{2+} . As shown in Fig. 22, Ca^{2+} -removal indeed blunted significantly the increase of annexin-V-binding following ochratoxin A treatment. However, the percentage of phosphatidylserine exposing erythrocytes still increased significantly following ochratoxin A treatment in the nominal absence of Ca^{2+} . Thus, Ca^{2+} -removal significantly blunted, but did not fully abrogate the ochratoxin A induced erythrocyte membrane scrambling.

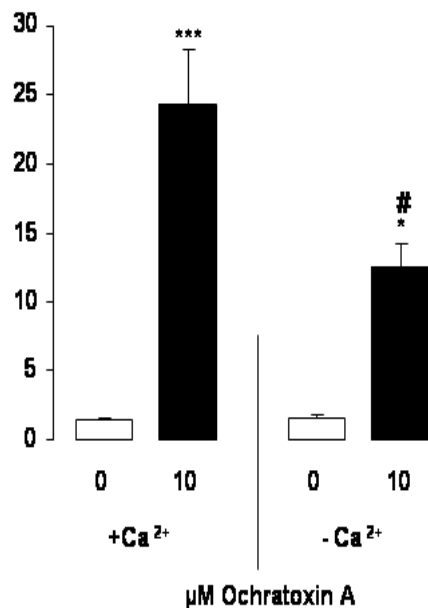


Fig. 22. Effect of Ca²⁺ withdrawal on ochratoxin A- induced annexin-V-binding.

Arithmetic means \pm SEM ($n = 4$) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bar) or with (black bars) ochratoxin A (10 μM) in the presence (left bars, +Calcium) and absence (right bars, -Calcium) of Ca²⁺. * ($p < 0.05$), *** ($p < 0.001$) indicates significant difference from the absence of ochratoxin A (ANOVA) # ($p < 0.05$) indicates significant difference from the respective value in the presence of Ca²⁺.

Additional experiments were performed to explore, whether ochratoxin A stimulates the formation of ceramide, which is known to enhance the Ca²⁺-sensitivity of cell membrane scrambling and thus to trigger eryptosis. Ceramide abundance at the erythrocyte surface was quantified utilizing FITC-labeled anti-ceramide antibodies. As shown in Fig. 23, ochratoxin A (10 μM) significantly increased ceramide formation. Additional experiments explored the effect of the sphingomyelinase inhibitor amitriptyline. Treatment with ochratoxin A significantly enhanced annexin V binding from $2.9 \pm 0.1\%$ ($n = 4$) to $26.4 \pm 1.5\%$ ($n = 4$) in the absence and to $31.8 \pm 0.2\%$ ($n = 4$) in the presence of amitriptyline (10 μM). Thus, amitriptyline did not significantly modify annexin binding following ochratoxin A treatment.

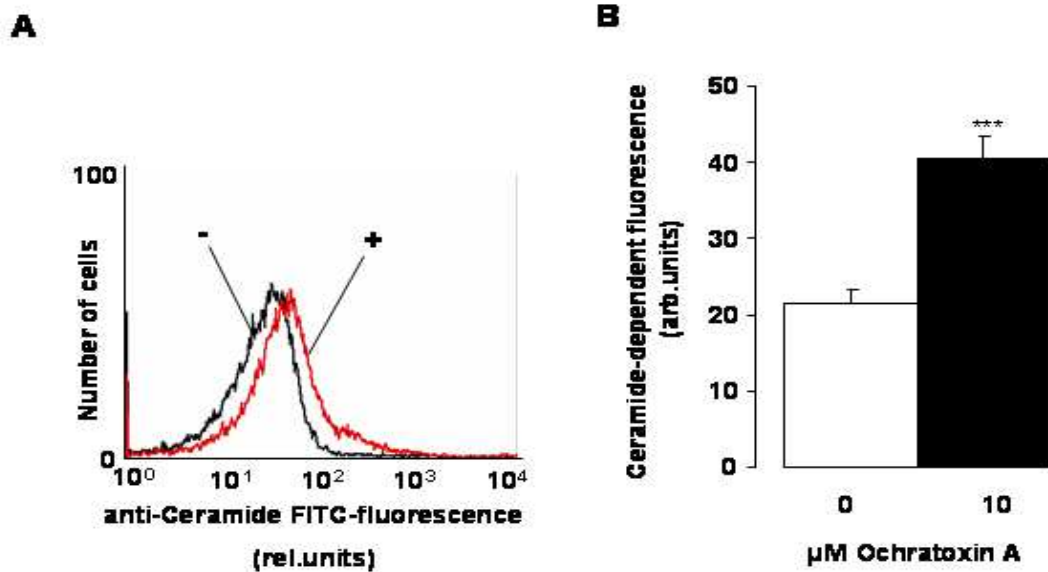


Fig. 23. Effect of ochratoxin A on ceramide formation.

A. Original histogram of anti-ceramide FITC-fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of ochratoxin A (10 μ M). **B.** Arithmetic means \pm SEM (n = 4) of ceramide abundance after a 48 h incubation in Ringer solution without (white bar) or with (black bars) ochratoxin A (10 μ M). *** (p<0.001) indicates significant difference from control (absence of ochratoxin A) (t test).

Further experiments explored whether or not ochratoxin A influences the energy status of erythrocytes. To this end, cytosolic ATP concentrations were determined. As a result, the ATP concentrations were virtually identical in the presence (2.7 ± 0.1 mM; n = 4) and absence (2.7 ± 0.1 mM; n = 4) of ochratoxin A. Thus, ochratoxin induced suicidal death of erythrocytes did not result from ATP depletion of erythrocytes.

Phosphatidylserine exposing erythrocytes are expected to adhere to the vascular wall. Thus, additional experiments were performed to test for an effect of ochratoxin A on adherence of erythrocytes to human umbilical vein endothelial cells (HUVEC) under *in vitro* flow conditions at arterial shear rates of 1200 s^{-1} . As illustrated in Fig. 24, treatment of erythrocytes with ochratoxin A (10 μ M) was followed by a marked increase of the number of erythrocytes adhering to HUVEC.

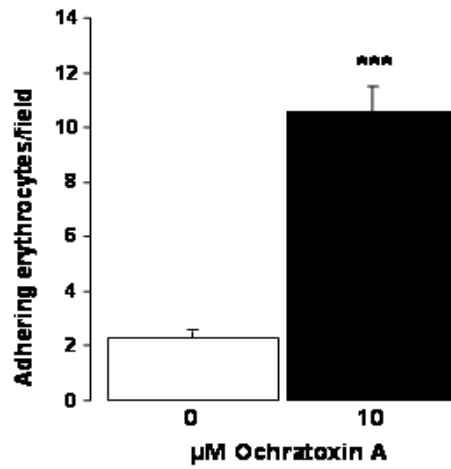


Fig. 24. Effect of ochratoxin A on adhesion of erythrocytes under flow conditions.

Arithmetic means \pm SEM (n = 4) of erythrocytes binding to human umbilical vein endothelial cells (HUVEC) under flow conditions following exposure for 48 hours to Ringer solution without (white bar), or with ochratoxin A (10 μ M) (black bar). *** (p<0.001) indicates statistically significant difference from the absence of ochratoxin A (10 μ M).

7.1.3. Zearalenone

The present study addressed the effect of zearalenone on suicidal erythrocyte death or eryptosis. The most important stimulator of eryptosis is increase of cytosolic Ca^{2+} activity. Thus, Fluo3 fluorescence was employed to estimate $[Ca^{2+}]_i$ in erythrocytes without or with prior exposure to zearalenone (1 – 50 μ M) in Ringer solution. Following incubation the erythrocytes were loaded with Fluo3-AM and Fluo3 fluorescence determined in FACS analysis. As illustrated in Fig. 25, a 48 hours exposure of human erythrocytes to zearalenone ($\geq 25 \mu$ M) was followed by a significant increase of Fluo3 fluorescence.

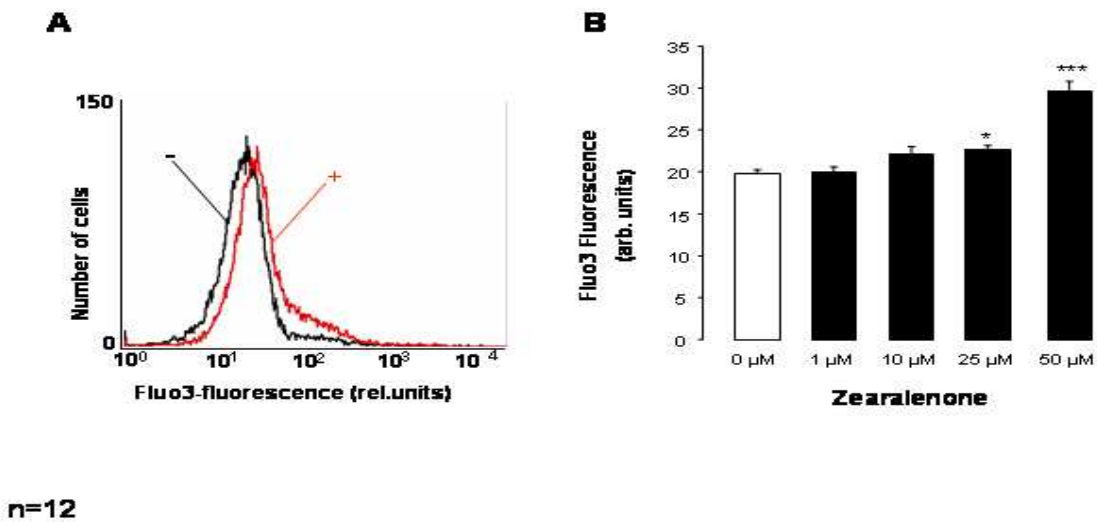


Fig. 25: Effect of zearalenone on erythrocyte cytosolic Ca^{2+} concentration

A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-) and with (+) presence of 50 μ M zearalenone. **B.** Arithmetic means \pm SEM (n = 12) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) zearalenone (1 – 50 μ M). * (p<0.05), ***(P<0.001) indicate significant difference from the absence of zearalenone (ANOVA).

An increase of $[Ca^{2+}]_i$ is expected to activate Ca^{2+} sensitive K^+ channels with subsequent KCl exit and thus cell shrinkage. In a next series of experiments erythrocyte volume was estimated from forward scatter in FACS analysis. As illustrated in Fig. 26, a 48 hours exposure to zearalenone was followed by a decrease of forward scatter, an effect reaching statistical significance at 10 μ M zearalenone concentration.

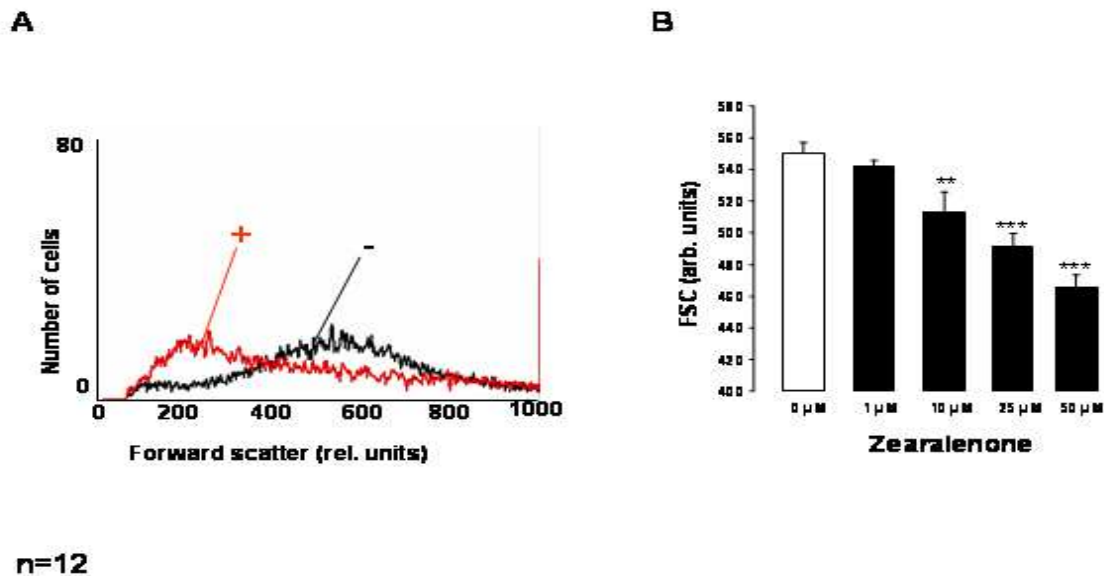


Fig. 26: Effect of zearalenone on erythrocyte forward scatter

A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (-) and with (+) presence of 50 μM zearalenone. **B.** Arithmetic means \pm SEM ($n = 12$) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) zearalenone (1 – 50 μM .) **($p < 0.01$), ***($p < 0.001$) indicates significant difference from the absence of zearalenone (ANOVA).

An increase of $[\text{Ca}^{2+}]_i$ is further expected to trigger cell membrane scrambling with phosphatidylserine exposure at the cell surface. Phosphatidylserine exposing erythrocytes were identified by determination of annexin-V-binding in FACS analysis. As shown in Fig. 27, a 48 h exposure to zearalenone increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at $\geq 25 \mu\text{M}$ zearalenone concentration.

Additional experiments were performed to explore whether zearalenone induces hemolysis, which was estimated by determination of hemoglobin in the supernatant. As illustrated in Fig, 28, the percentage of hemolysed erythrocytes increased slightly but significantly following exposure of erythrocytes for 48 h to zearalenone.

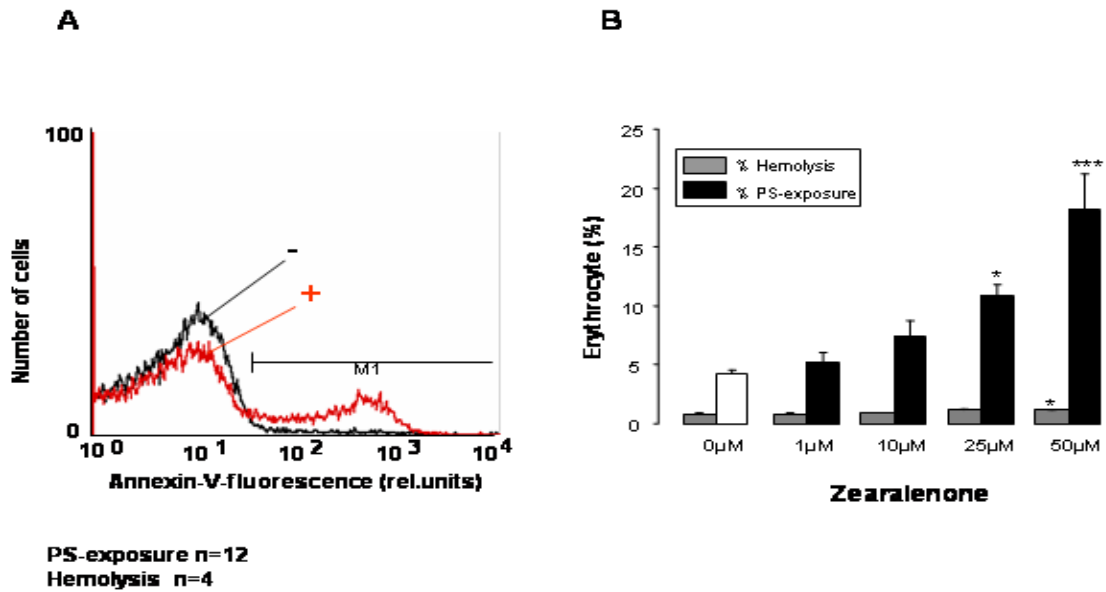
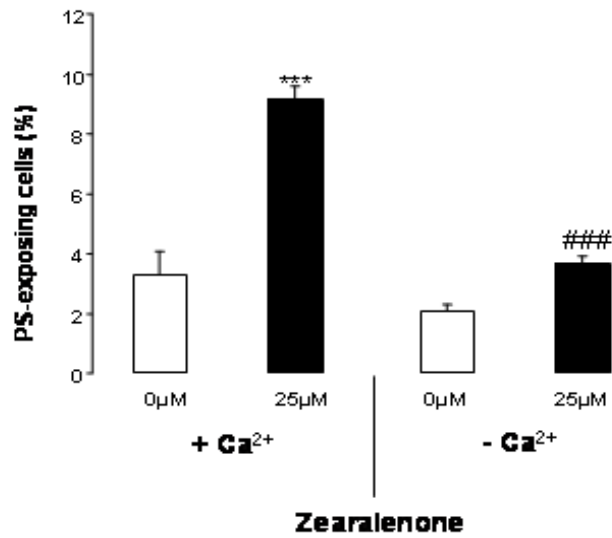


Fig. 28: Effect of zearalenone on phosphatidylserine exposure and hemolysis

A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (-) and with (+) presence of 50 μM zearalenone. **B.** Arithmetic means \pm SEM ($n = 12$) of erythrocyte annexin-V-binding following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of zearalenone (1 – 50 μM). For comparison, arithmetic means \pm SEM ($n = 4$) of the percentage of hemolysis is shown as grey bars. * ($p < 0.05$), *** ($P < 0.001$) indicate significant difference from the absence of zearalenone (ANOVA).

In order to quantify the contribution of Ca^{2+} entry from the extracellular space for the triggering of eryptosis, erythrocytes were exposed to 25 μM zearalenone for 48 hours in either the presence of 1 mM extracellular Ca^{2+} or in the absence of extracellular Ca^{2+} and presence of the Ca^{2+} chelator EGTA (1 mM). As shown in Fig. 29, the effect of zearalenone on annexin-V-binding was significantly decreased in the nominal absence of Ca^{2+} . In the absence of extracellular Ca^{2+} the percentage annexin V binding erythrocytes still tended to be higher following zearalenone treatment than without zearalenone treatment, a difference, however, not reaching statistical significance.



n=4

Fig. 29: Effect of Ca²⁺ withdrawal on zearalenone- induced annexin-V-binding

Arithmetic means \pm SEM (n = 4) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 25 μ M zearalenone in the presence (left bars, + Calcium) and absence (right bars, - Calcium) of calcium. *** (p<0.01) indicates significant difference from the absence of zearalenone (ANOVA) ###(p<0.001) indicates significant difference from the respective values in the presence of Ca²⁺.

7.2. Stimulation of suicidal death of erythrocytes by phytochemicals

7.2.1. Withaferin A

A hallmark of eryptosis is cell shrinkage which is apparent from a decrease of forward scatter. In order to test, whether withaferin A exposure leads to cell shrinkage, forward scatter was determined in FACS analysis. As shown in Fig.30, treatment of human erythrocytes for 48 hours with withaferin A was followed by a decrease of forward scatter, an effect reaching statistical significance at 5 μM concentration.

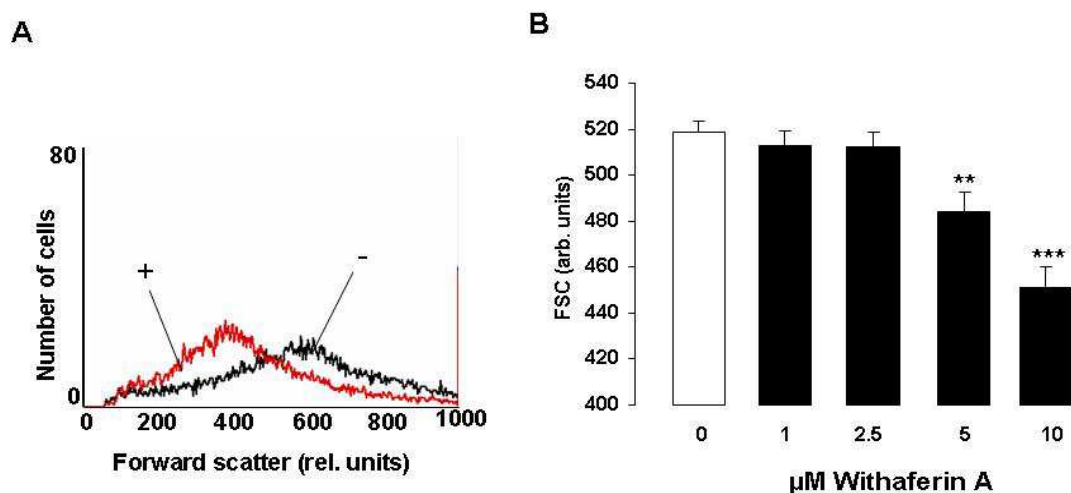


Fig. 30: Effect of withaferin A on erythrocyte forward scatter

A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (-) and with (+) presence of 10 μM withaferin A. **B.** Arithmetic means \pm SEM ($n = 12$) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) withaferin A (1 – 10 μM). **($p < 0.01$), *** ($p < 0.001$) indicates significant difference from the absence of withaferin A (ANOVA).

The second hallmark of eryptosis is cell membrane scrambling with phosphatidylserine exposure at the cell surface. In order to test whether withaferin A treatment triggers cell membrane scrambling, phosphatidylserine exposing erythrocytes were identified by measurement of annexin-V-binding in FACS analysis. As illustrated in Fig. 31, a 48 h exposure to withaferin A increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 5 μM concentrations. To rule out an effect of solvent, annexin-V-binding was estimated in erythrocytes exposed to DMSO (0.2%). As a result, annexin-V-binding was not

significantly different following incubation in the absence ($2.3 \pm 0.0\%$, $n = 4$). and presence ($2.4 \pm 0.1\%$, $n = 4$) of DMSO (0.2%).

Further experiments explored whether treatment with withaferin A is followed by hemolysis, which was calculated from hemoglobin release into the supernatant. As shown in Fig. 32, exposure of erythrocytes for 48 h to withaferin A increased the hemoglobin concentration in the supernatant, an effect reaching statistical significance at 5 μM (Fig. 32B). The percentage of hemolysed erythrocytes was, however, ~ 5 -fold smaller than the percentage of phosphatidylserine exposing cells.

In order to correlate cell shrinkage with phosphatidylserine exposure, a dot plot analysis of the histograms was performed. As shown in Fig. 32C and 32D, the percentage of annexin-V positive erythrocytes is significantly enhanced upon treatment with withaferin A in both sub-populations of cells with lower (<400 , arb. units) and higher cell volumes (>400 , arb. units).

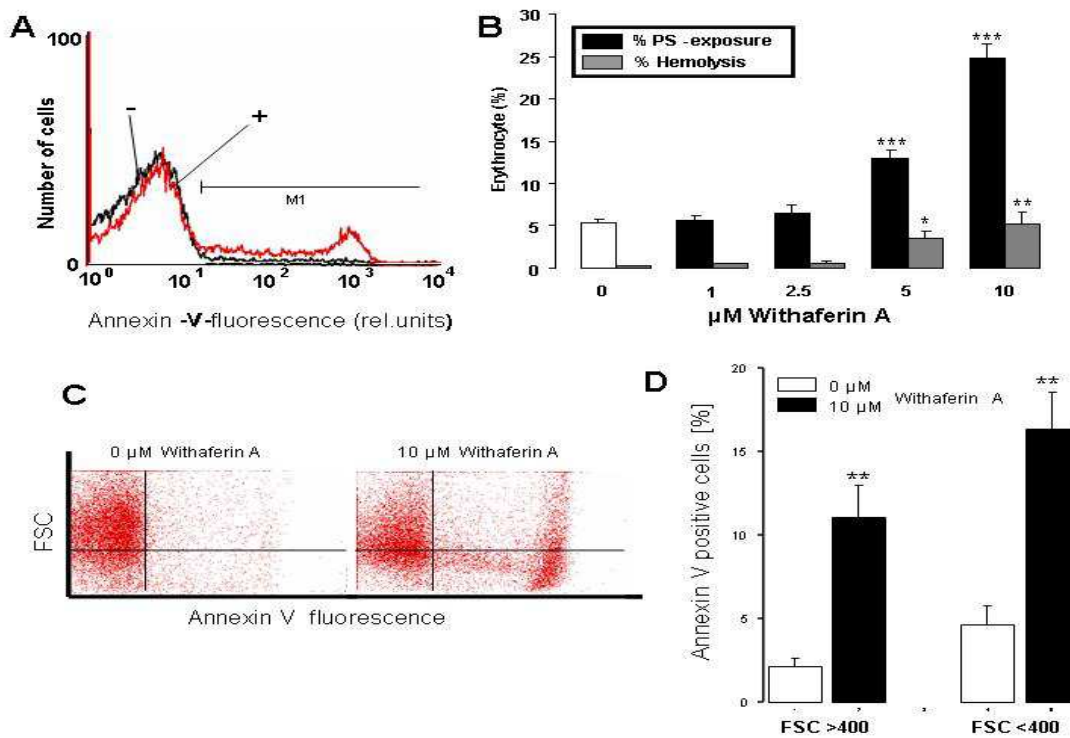


Fig. 32: Effect of withaferin A on phosphatidylserine exposure and hemolysis

A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (-) and with (+) presence of 10 μM withaferin A. **B.** Arithmetic means \pm SEM of erythrocyte annexin-V-binding ($n = 12$) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of withaferin A (1 – 10 μM). For comparison, arithmetic means \pm SEM ($n = 4$) of the percentage of hemolysis is shown as grey bars. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) indicates significant difference from the absence of withaferin A (ANOVA). **C.** Original dot plots of FSC versus Annexin-V fluorescence of erythrocytes following exposure for 48 h to Ringer solution without (left panel) and with (right panel) the presence of 10 μM withaferin A. **D.** Arithmetic means \pm SEM of annexin-V positive erythrocytes ($n = 4$) with decreased cell volume (< 400 , arb. units) and increased cell volume (> 400 , arb. units) following incubation for 48 h in Ringer solution without (white bars) or with (black bars) presence of withaferin A (10 μM). ** ($p < 0.01$) indicates significant difference from the absence of withaferin A (t test).

Both, cell shrinkage and cell membrane scrambling could result from increase of cytosolic Ca^{2+} activity. In order to determine cytosolic Ca^{2+} activity, erythrocytes were loaded with Fluo 3 and the Fluo3 fluorescence was determined by FACS analysis. As illustrated in Fig. 33, treatment of human erythrocytes with withaferin A increased Fluo3 fluorescence, an effect reaching statistical significance at 5 μM withaferin A concentration. Thus, withaferin A treatment increases cytosolic Ca^{2+} concentration in a subpopulation of human erythrocytes. A dot plot analysis of the histograms was performed to correlate cell shrinkage with cytosolic Ca^{2+} activity. As shown in Fig. 33C and 33D, the percentage of Fluo3 positive erythrocytes is significantly enhanced upon treatment with withaferin A in both sub-populations of cells with lower (< 400 , arb. units) and higher cell volumes (> 400 , arb. units).

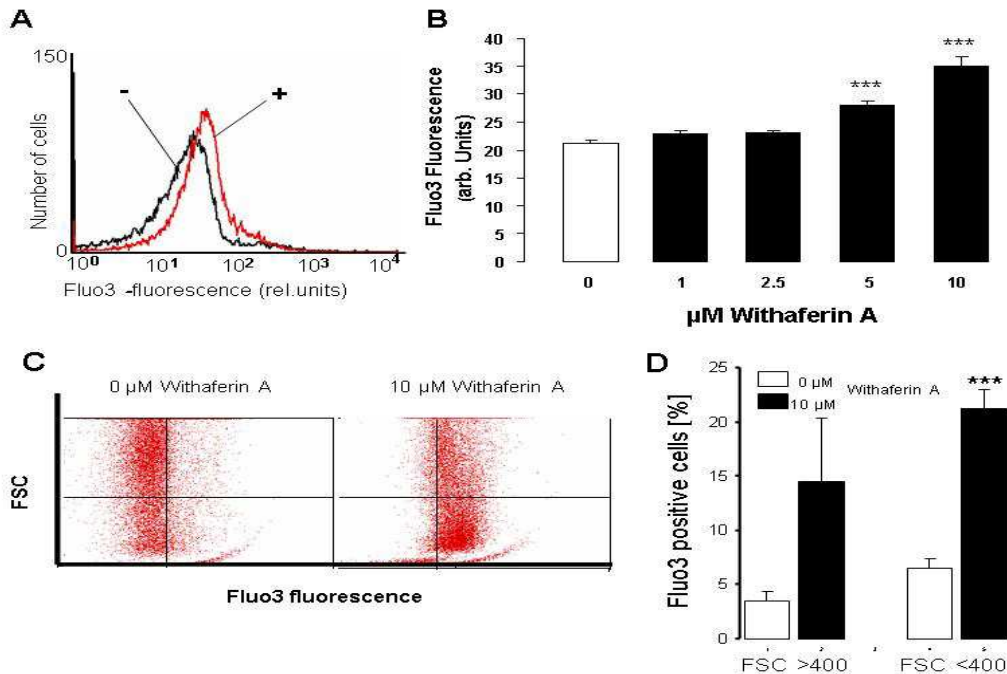


Fig. 33: Effect of withaferin A on erythrocyte cytosolic Ca^{2+} concentration

A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-) and with (+) presence of 10 μM withaferin A. **B.** Arithmetic means \pm SEM ($n = 12$) of the normalized geo means of Fluo3 fluorescence in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) withaferin A (1 – 10 μM). *** ($p < 0.001$) indicates significant difference from the absence of withaferin A (ANOVA). **C.** Original dot plots of FSC versus Fluo3 fluorescence of erythrocytes following exposure for 48 h to Ringer solution without (left panel) and with (right panel) the presence of 10 μM withaferin A. **D.** Arithmetic means \pm SEM of Fluo3 positive erythrocytes ($n = 4$) with decreased cell volume (< 400 , arb. units) and increased cell volume (> 400 , arb. units) following incubation for 48 h in Ringer solution without (white bars) or with (black bars) presence of withaferin A (10 μM). *** ($p < 0.001$) indicates significant difference from the absence of withaferin A (t test).

In order to test, whether the increase of cytosolic Ca^{2+} activity was required for the stimulation of cell membrane scrambling by withaferin A, erythrocytes were exposed to 10 μM withaferin A in the presence or in the nominal absence of extracellular Ca^{2+} . As shown in Fig. 34A, the effect of withaferin A on annexin-V-binding was significantly blunted in the nominal absence of Ca^{2+} . However, even in the nominal absence of Ca^{2+} , withaferin A significantly stimulated annexin-V-binding. Accordingly, Ca^{2+} entry and subsequent increase of cytosolic Ca^{2+} activity contributed to but did not fully account for the stimulation of erythrocyte membrane scrambling following treatment with withaferin A. To test, whether erythrocyte membrane scrambling was influenced by Mg^{2+} , further experiments were performed in the presence and absence of Mg^{2+} and in the presence of EGTA and absence of Ca^{2+} . As a result, in this series the treatment with withaferin A (10 μM) increased the percentage of annexin-V-binding cells from $3.4 \pm 0.2\%$ to $12.0 \pm 0.7\%$ ($n = 4$) in the presence of Mg^{2+} and from 3.5 ± 0.2 to $12.2 \pm 0.9\%$ ($n = 4$) in the absence of Mg^{2+} , values not significantly different. Similar to extracellular Ca^{2+} removal, the non selective cation channel blocker amiloride (1 mM) significantly blunted withaferin-triggered phosphatidylserine exposure (Fig. 34B).

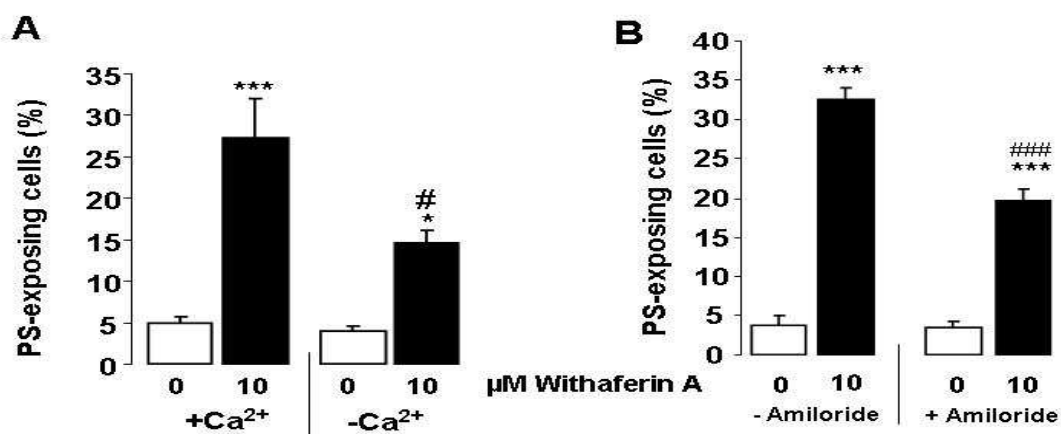


Fig. 34: Role of Ca^{2+} in withaferin A- induced annexin-V-binding

A. Arithmetic means \pm SEM ($n = 4$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 10 μM withaferin A in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of calcium. * ($p < 0.05$), *** ($p < 0.001$) indicates significant difference from the absence of withaferin A (ANOVA), # ($p < 0.05$) indicates significant difference from the respective values in the presence of Ca^{2+} . **B.** Arithmetic means \pm SEM ($n = 4$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 10 μM withaferin A in the presence (left bars, -amiloride) and absence (right bars, +amiloride) of amiloride (1 mM). *** ($p < 0.001$) indicates significant difference from the absence of withaferin A (ANOVA), ### ($p < 0.001$) indicates significant difference from the respective values in the absence of amiloride.

As a next step, the effect of withaferin A on oxidative stress was tested by measuring 2',7' dichlorodihydrofluorescein diacetate (DCFDA) fluorescence. Exposure of erythrocytes to withaferin A (10 μM) was followed by a significant increase in DCFDA-dependent fluorescence pointing to oxidative stress (Fig. 35A and 35B). To examine the role of oxidative stress in withaferin A-induced eryptosis, the effect of the antioxidant scavenger N-acetyl-L-cysteine (NAC) was tested. As shown in Fig. 35C, 1mM NAC significantly blunted the increase in phosphatidylserine-exposing erythrocytes induced by withaferin A. These observations point to oxidative stress in withaferin A-induced suicidal erythrocyte death.

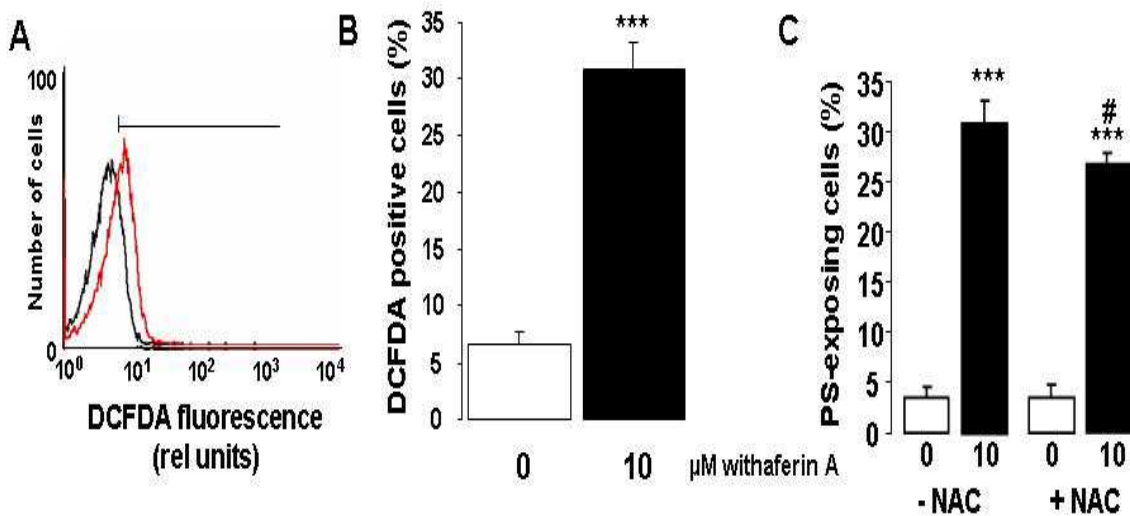


Fig. 35: Effect of withaferin A on generation of reactive oxygen species

A. Original histogram of DCFDA-positive erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) 10 μM withaferin A. **B.** Arithmetic means \pm SEM ($n = 4$) of the percentage of DCFDA-positive erythrocytes following incubation for 48 h to Ringer solution without (white bar) or with (black bar) 10 μM withaferin A. *** ($p < 0.001$) indicates significant difference from the absence of withaferin A (ANOVA). **C.** Arithmetic means \pm SEM ($n = 4$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 10 μM withaferin A in the absence (left bars, -NAC) and presence (right bars, +NAC) of 1 mM N-acetyl-cysteine (NAC). *** ($p < 0.001$) indicates significant difference from

the absence of withaferin A (ANOVA), # ($p < 0.05$) indicates significant difference from the respective values in the absence of NAC (ANOVA).

The sensitivity of eryptosis to cytosolic Ca^{2+} activity could be enhanced by ceramide. An additional series of experiments thus explored the effect of withaferin A treatment on the formation of ceramide. Ceramide abundance was determined utilizing FITC-labeled anti-ceramide antibodies. As illustrated in Fig. 36A and 36B, withaferin A significantly increased ceramide-dependent fluorescence.

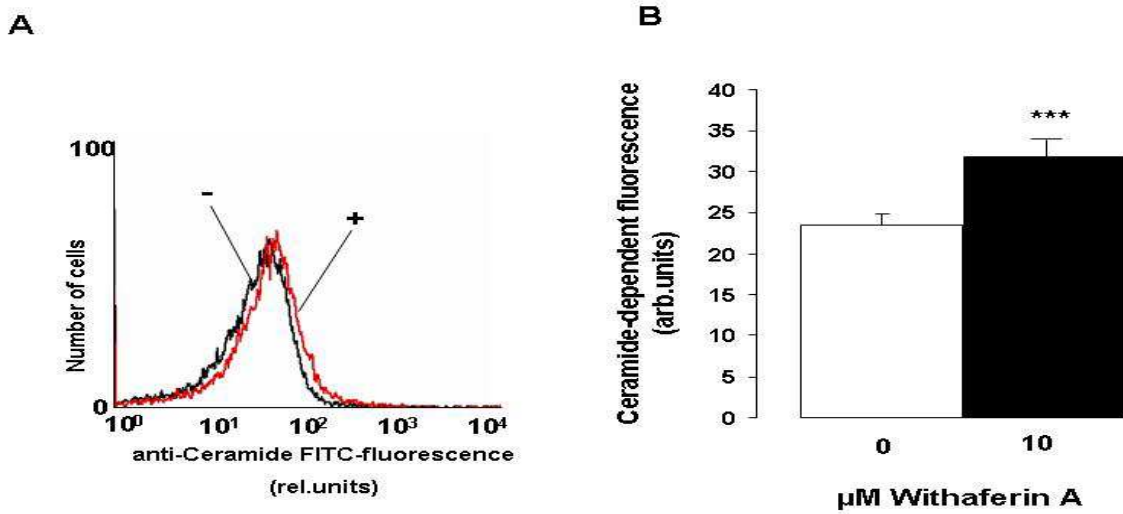


Fig. 36. Effect of withaferin A on ceramide formation.

A. Original histogram of ceramide-dependent fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-) and with (+) presence of 10 μ M withaferin. **B.** Arithmetic means \pm SEM ($n = 8$) of ceramide abundance after a 48 h incubation in Ringer solution without (white bar) or with (black bar) withaferin A (10 μ M). *** ($p < 0.001$) indicates significant difference from control (absence of withaferin A) (ANOVA).

7.2.2. Oridonin

Cytosolic Ca^{2+} concentration was estimated utilizing Fluo 3 fluorescence. As illustrated in Fig. 37, exposure of human erythrocytes to Ringer solution containing oridonin was followed by an increase of cytosolic Ca^{2+} concentration, an effect reaching statistical significance at an oridonin concentration of 10 μM .

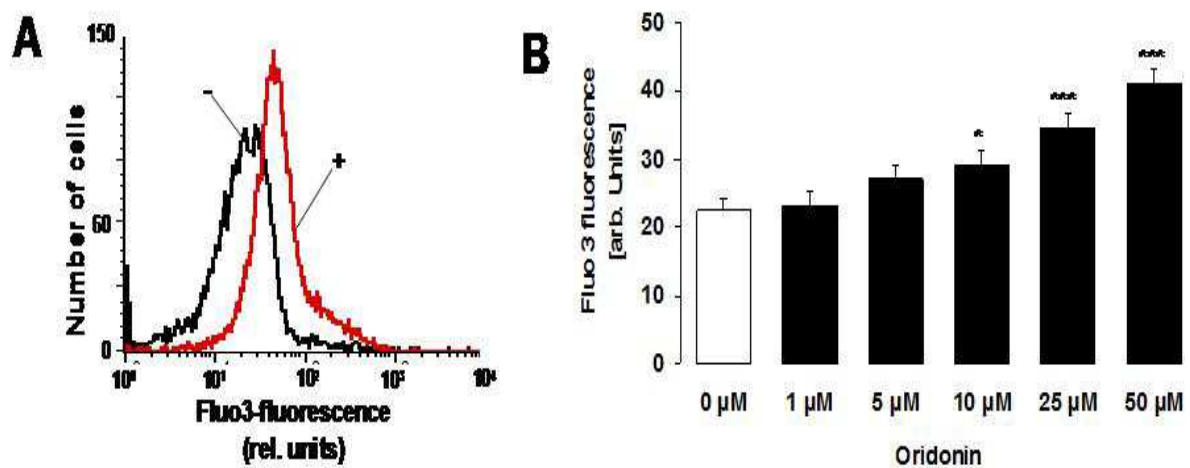


Fig. 37: Effect of oridonin on erythrocyte cytosolic Ca^{2+} concentration

A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of 50 μM oridonin. **B.** Arithmetic means \pm SEM ($n = 8$) of the geo means (geometric mean of the histogram in arbitrary units) of Fluo3 fluorescence in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) 1-50 μM oridonin. * ($p < 0.05$), *** ($p < 0.001$) indicate significant difference from the absence of oridonin (ANOVA).

As Ca^{2+} activates the Ca^{2+} sensitive K^{+} channels, an increase of cytosolic Ca^{2+} concentration is expected to result in exit of KCl with osmotically obliged water thus leading to cell shrinkage. Accordingly, forward scatter was determined to analyze the effect of oridonin on cell volume. As demonstrated in Fig. 38, exposure of erythrocytes for 48 hours to Ringer solution containing oridonin was indeed followed by a decrease of forward scatter, an effect reaching statistical significance at an oridonin concentration of 25 μM .

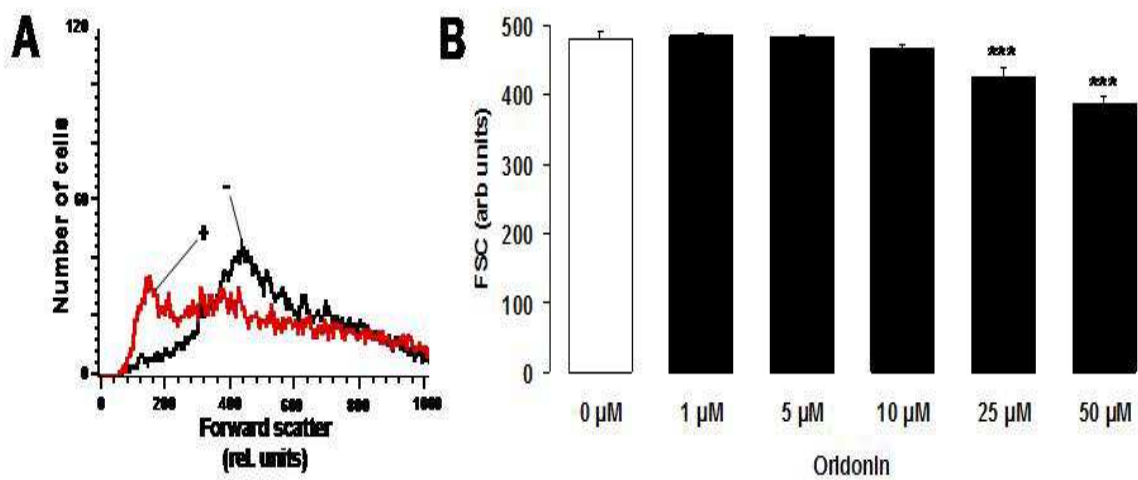


Fig. 39: Effect of oridonin on erythrocyte forward scatter

A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of 50 μM oridonin. **B.** Arithmetic means \pm SEM ($n = 8$) of the erythrocyte forward scatter following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) 1-50 μM oridonin. *** ($p < 0.001$) indicate significant difference from the absence of oridonin (ANOVA).

Ca^{2+} further stimulates cell membrane scrambling with subsequent phosphatidylserine exposure at the cell surface. Annexin V binding was employed to identify phosphatidylserine exposing erythrocytes. Annexin V binding in the absence and presence of 50 μM oridonin was visualized by confocal imaging (Fig. 40). The number of annexin V positive erythrocytes was indeed increased following oridonin treatment.

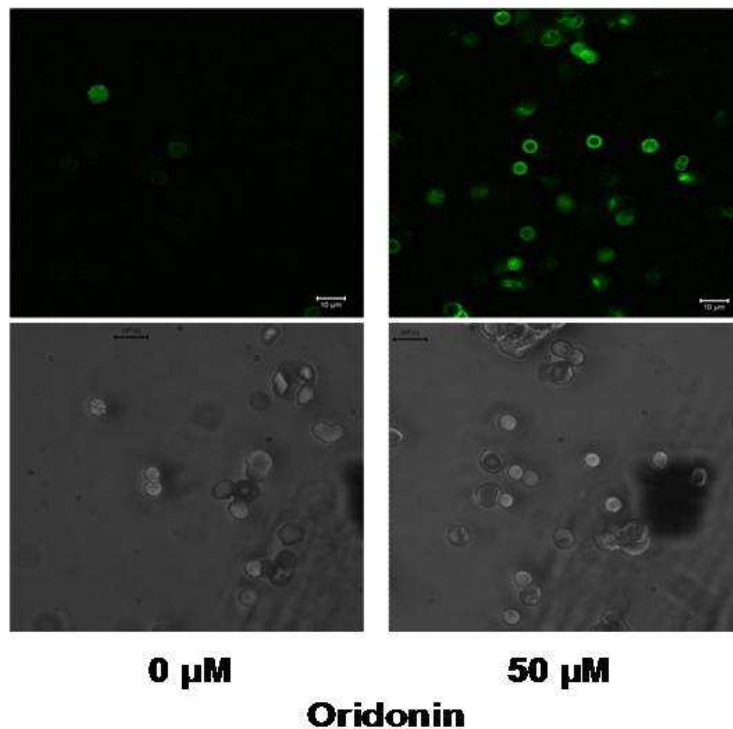


Fig. 40: Confocal images of PS-exposing erythrocytes with or without prior oridonin treatment

Confocal microscopy of FITC-dependent fluorescence (upper panels) and light microscopy (lower panels) of human erythrocytes stained with FITC-conjugated Annexin-V-Fluos following 48 hours incubation in Ringer solution without (left panels) and with (right panels) 50 μM oridonin.

FACS analysis was employed to quantify annexin V binding. As illustrated in Fig. 41A and 41B, exposure of erythrocytes for 48 hours to Ringer solution containing oridonin increased the percentage of annexin V binding erythrocytes, an effect reaching statistical significance at 25 μM oridonin.

To determine hemolysis, the hemoglobin release into the supernatant was determined following exposure of the erythrocytes to Ringer solution without or with 1-50 μM oridonin. As shown in Fig. 41B, the exposure to oridonin was followed by hemolysis, an effect reaching statistical significance at 10 μM oridonin. The percentage of hemolytic erythrocytes remained, however, one order of magnitude smaller than the percentage of annexin V binding erythrocytes.

An additional series of experiments explored whether oridonin-induced cell membrane scrambling was dependent on the presence of Ca^{2+} . To this end, erythrocytes were exposed to oridonin in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 41C, the

effect of oridonin on annexin V-binding was blunted, but not abolished in the nominal absence of Ca^{2+} . In a further series of experiments, the Ca^{2+} permeable cation channels were inhibited by amiloride (1 mM). As shown in Fig. 41D, the addition of amiloride (1 mM) blunted, but did not abolish the effect of oridonin on annexin V-binding.

Collectively, the experiments suggest that exposure of erythrocytes to oridonin increased intracellular Ca^{2+} activity, which in turn contributed to subsequent cell shrinkage and cell membrane scrambling. The residual cell membrane scrambling in the nominal absence of Ca^{2+} or in the presence of the cation channel blocker amiloride pointed, however, to an additional mechanism in the triggering of eryptosis following exposure to oridonin.

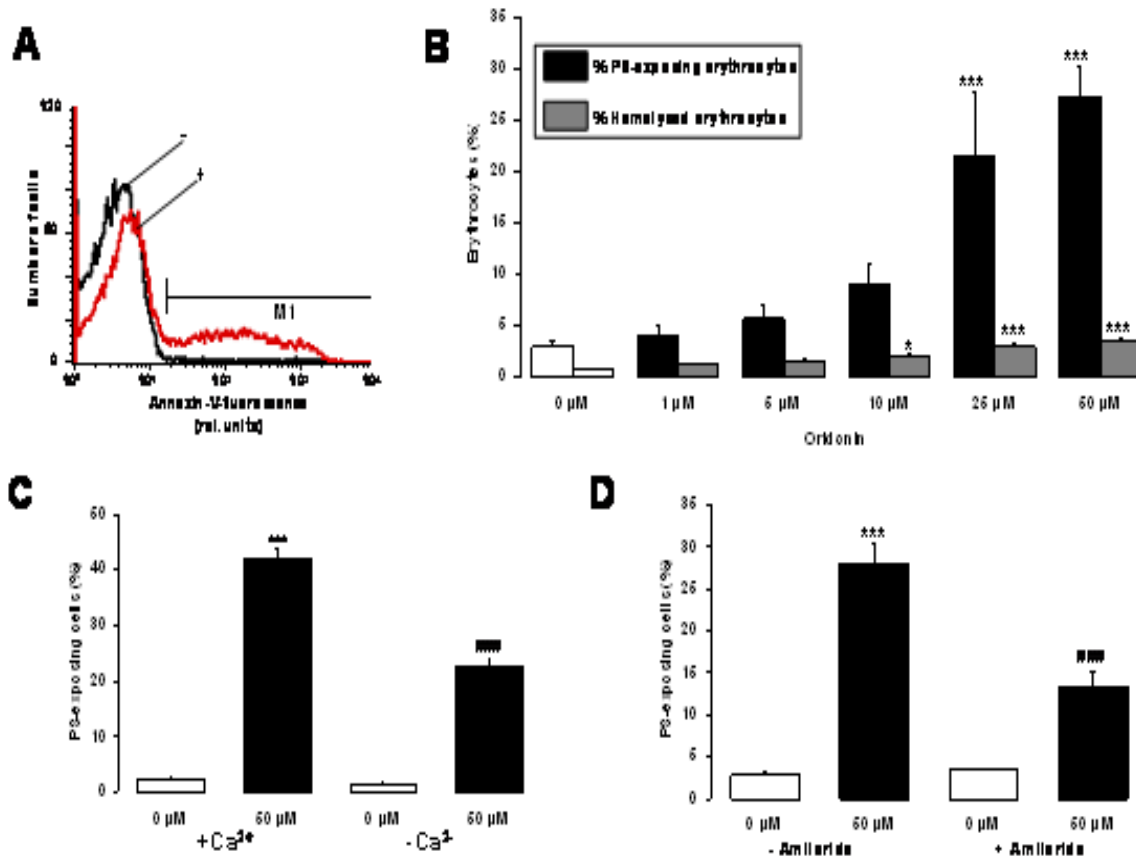


Fig. 41: Effect of oridonin on phosphatidylserine exposure

A. Original histogram of annexin V binding of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of 50 μM oridonin. **B.** Arithmetic means ± SEM (n = 8) of erythrocyte annexin V binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of 1-50 μM oridonin. For comparison, arithmetic means ± SEM (n = 4) of the percentage of hemolysis are shown as grey bars. *(p < 0.05), *** (p < 0.001) indicate significant difference from the absence of

oridonin (ANOVA). C. Arithmetic means \pm SEM (n = 8) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bar) or with (black bars) 50 μ M oridonin in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of calcium. *** (p<0.001) indicate significant difference from the absence of oridonin (ANOVA), ### (p<0.001) indicates significant difference from the respective values in the presence of Ca^{2+} . D. Arithmetic means \pm SEM (n = 5) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bar) or with (black bars) 50 μ M oridonin in the absence (left bars) and presence (right bars) of 1 mM amiloride. *** (p<0.001) indicate significant difference from the absence of oridonin (ANOVA), ### (p<0.001) indicates significant difference from the respective values in the absence of 1 mM amiloride.

As eryptosis is stimulated by energy depletion, cytosolic ATP concentration was determined in erythrocytes incubated for 48 hours without or with oridonin. For comparison, the ATP concentration was determined in erythrocytes exposed to glucose-free Ringer. As shown in Fig. 42, exposure to oridonin did not significantly modify cytosolic ATP concentration. In contrast, glucose withdrawal was followed by the expected decrease of ATP concentration.

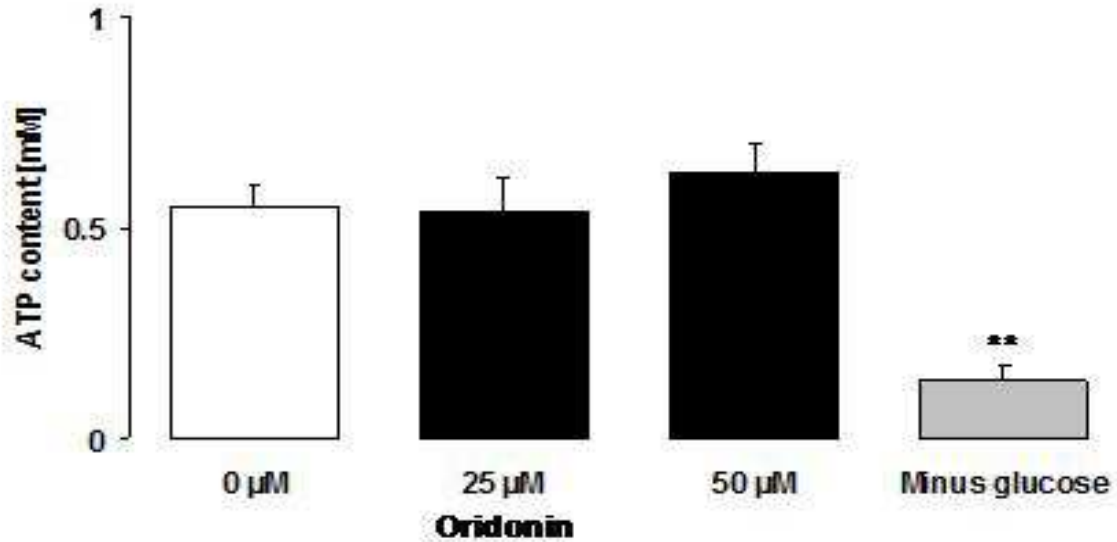


Fig. 42: Effect of oridonin on erythrocyte cytosolic ATP content

Arithmetic means \pm SEM (n = 4) of the ATP concentration after a 48 hours incubation in Ringer solution without (white bar) or with (black bars) oridonin at the indicated concentrations, or in glucose-depleted Ringer solution (grey bar, minus glucose) ** (p<0.01) indicates significant difference from control (absence of oridonin and presence of glucose) (ANOVA).

Phosphatidylserine exposure and cell shrinkage are further known to be triggered by ceramide. Accordingly, additional experiments were performed to elucidate whether exposure to oridonin increases ceramide formation in erythrocytes. According to binding of FITC labelled anti-

ceramide antibodies, ceramide formation was indeed increased by oridonin exposure, an effect reaching statistical significance at 10 μ M oridonin concentration (Fig. 43).

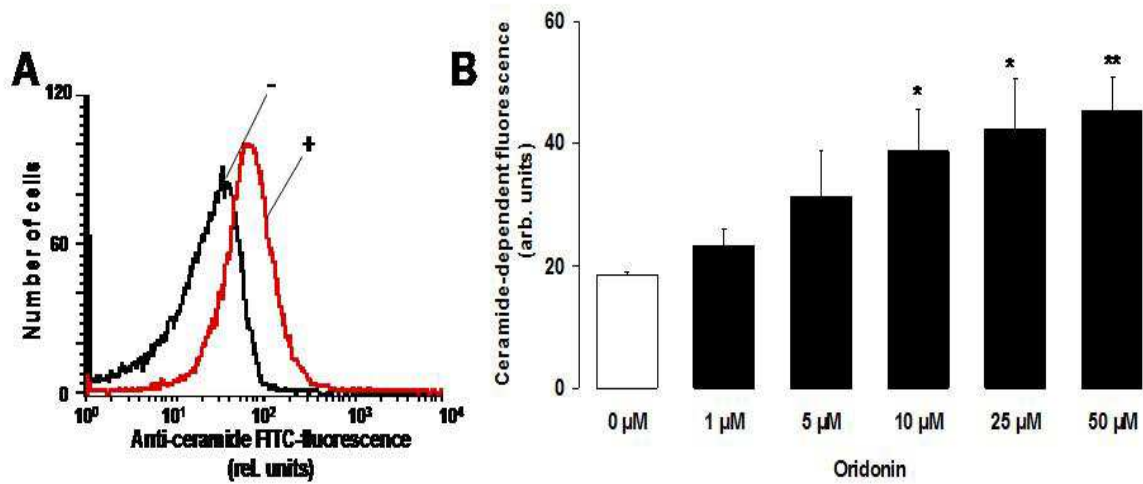


Fig. 43: Effect of oridonin on ceramide formation

A. Original histogram of annexin V binding of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of 50 μ M oridonin. **B.** Arithmetic means \pm SEM (n = 4) of ceramide abundance in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of 1-50 μ M oridonin. *(p<0.05), ** (p<0.01) indicate significant difference from the absence of oridonin (ANOVA).

7.2.3. Dicoumarol

Cytosolic Ca^{2+} concentration in human erythrocytes was determined utilizing Fluo 3 fluorescence in FACS analysis. As illustrated in Fig. 44A and 44B, a 48 hours exposure to dicoumarol increased cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), an effect reaching statistical significance at 20 μM dicoumarol concentration. At higher concentration (30 μM) dicoumarol increased $[\text{Ca}^{2+}]_i$ (by $17.0 \pm 3.3\%$, $n = 4$) within 1 hour.

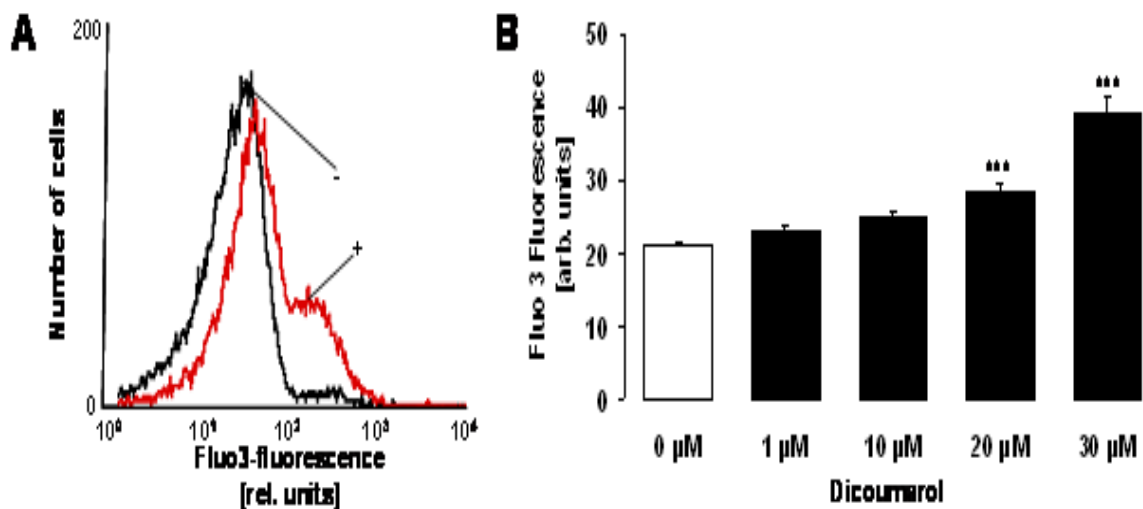


Fig. 44: Effect of dicoumarol on erythrocyte cytosolic Ca^{2+} concentration

A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer's solution without (-, black line) and with (+, red line) the presence of 30 μM dicoumarol. **B.** Arithmetic means \pm SEM ($n = 16$) of the geo means (geometric mean of the histogram in arbitrary units) of Fluo3 fluorescence in erythrocytes exposed for 48 hours to Ringer's solution without (white bar) or with (black bars) 1-30 μM dicoumarol. *** indicates significant difference ($p < 0.001$) from the respective value in the absence of dicoumarol (ANOVA).

Whole cell patch-clamp experiments were performed to elucidate whether dicoumarol influences cation ion channel activity. Replacing Ringer bath solution with Na-gluconate markedly increased, whereas replacing Na^+ with NMDG or Ca^{2+} decreased currents in untreated control cells when recorded with Na-gluconate in the pipette solution. In addition, substitution of Na^+ by the larger and less permeable cation NMDG^+ shifted the reversal potentials of the current-voltage-relationships (I/V) from ~ 0 mV in Ringer bath (Fig. 45 A) to about -60 mV (Fig. 45 B). The data suggest the following rank of cation perm selectivity of the channels in control cells: $\text{Na}^+ > \text{Ca}^{2+} \gg \text{NMDG}^+$. As illustrated in Fig. 45, dicoumarol (30 μM) treatment indeed significantly enhanced the cation conductance irrespective to the cation used in the bath solutions. The cation conductance following Cl-removal was also found to be more than 2 times higher in cells treated

with dicoumarol than in control cells (0.482 nS for dicoumarol-treated and 0.213 nS for control cells, respectively).

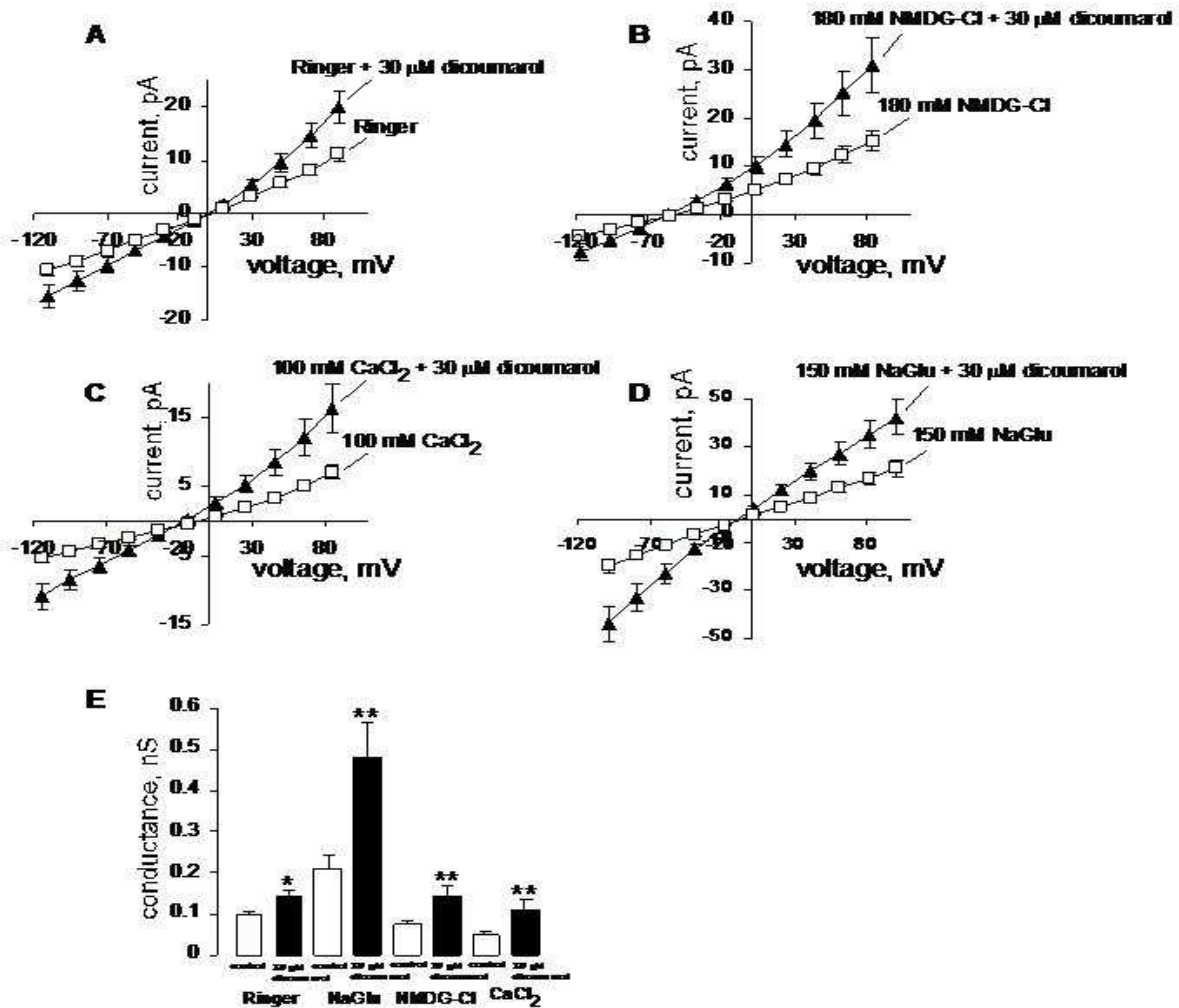


Fig. 45: Effect of dicoumarol on cation channels in human erythrocytes.

A. Mean (\pm SEM) of I/V relationships in erythrocytes incubated for 3-6 hours at 37 °C in Ringer bath solution in the absence (open squares, n = 18) or presence (closed triangles, n = 11) of 30 μ M dicoumarol. Currents were recorded with the Na-gluconate pipette and Ringer (\pm 30 μ M dicoumarol) bath solutions. **B.** Mean (\pm SEM) of I/V relationships (as in A) for the currents recorded in 180 mM NMDG-Cl bath solution (n = 18 for control and n = 11 for 30 μ M dicoumarol treated cells). **C.** Mean (\pm SEM) of I/V relationships (as in A) for the currents recorded in 100 mM CaCl₂ bath solution (n = 16 for control and n = 10 for 30 μ M dicoumarol treated cells). **D.** Mean (\pm SEM) of I/V relationships (as in A) for the currents recorded in 150 mM Na-gluconate bath solution (n = 16 for control and n = 11 for 30 μ M dicoumarol treated cells). **E.** Mean (\pm SEM, n = 11-18) conductance (as calculated from the data in (A, B) for the inward currents by linear regression) in the absence (open bars) and presence (closed bar) of 30 μ M dicoumarol. *, ** indicate significant difference (p<0.05, p<0.001) from the respective value in the absence of dicoumarol (t-test).

An increase of cytosolic Ca^{2+} concentration were expected to stimulate Ca^{2+} sensitive K^+ channels leading to K^+ exit, hyperpolarisation, Cl^- exit and thus to cell shrinkage by cellular loss of KCl and osmotically obliged water. Cell volume was thus determined utilizing forward scatter in FACS analysis. As shown in Fig. 46A and 46B, a 48 hours exposure of erythrocytes to dicoumarol decreased the forward scatter, an effect reaching statistical significance at 10 μM dicoumarol. Thus, dicoumarol exposure was followed by cell shrinkage.

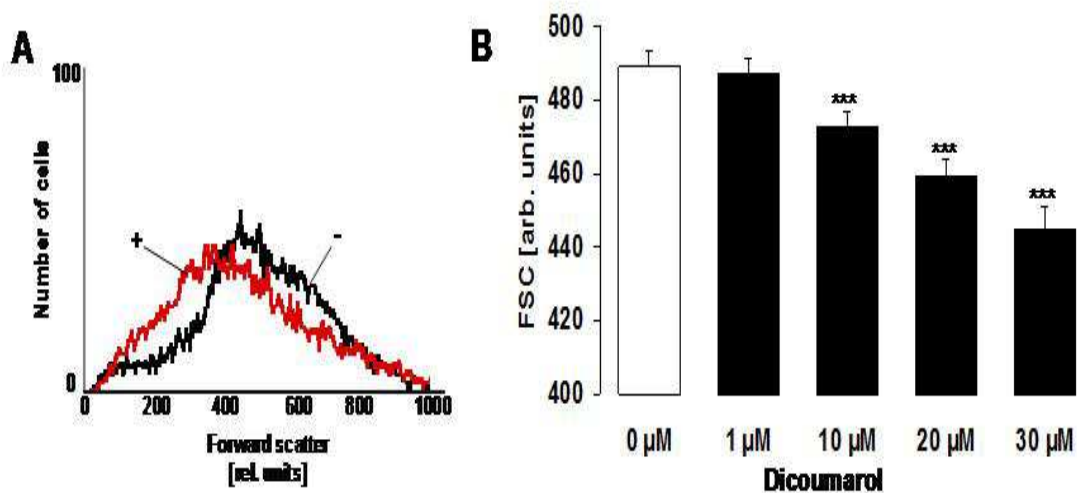


Fig. 46: Effect of dicoumarol on erythrocyte forward scatter

A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer's solution without (-, black line) and with (+, red line) the presence of 30 μM dicoumarol. **B.** Arithmetic means \pm SEM ($n = 16$) of the erythrocyte forward scatter following incubation for 48 hours in Ringer's solution without (white bar) or with (black bars) 1-30 μM dicoumarol. *** ($p < 0.001$) indicate significant difference from the absence of dicoumarol (ANOVA).

An increase of cytosolic Ca^{2+} concentration was further expected to trigger cell membrane scrambling with phosphatidylserine exposure at the cell surface. Phosphatidylserine exposing erythrocytes were thus identified by annexin V binding. As apparent from Fig. 47, dicoumarol exposure increased the percentage of annexin V binding erythrocytes. The percentage of phosphatidylserine exposing erythrocytes was quantified in FACS analysis.

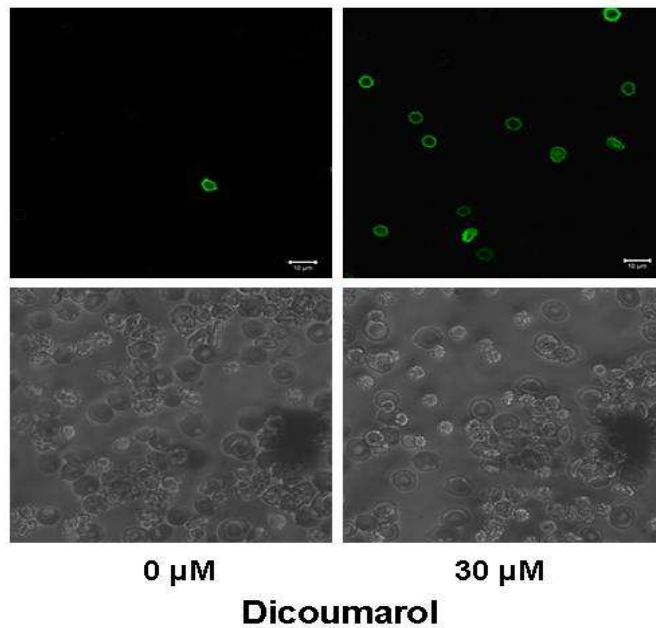


Fig. 47: Confocal images of PS-exposing erythrocytes exposed to dicoumarol

Confocal microscopy of FITC-dependent fluorescence (upper panels) and light microscopy (lower panels) of human erythrocytes stained with FITC-conjugated Annexin-V-Fluos following 48 hours incubation in Ringer's solution without (left panels) and with (right panels) 30 μM dicoumarol.

As illustrated in Fig. 48A and 48B, the percentage of annexin V binding erythrocytes was higher following a 48 hours exposure of erythrocytes to Ringer's solution containing dicoumarol than following a 48 hours exposure of erythrocytes to Ringer's solution without dicoumarol. The difference of the percentage annexin V binding erythrocytes between the presence and absence of dicoumarol reached statistical significance at $\geq 10 \mu\text{M}$ dicoumarol.

Exposure to dicoumarol was further followed by hemolysis, reflected by hemoglobin release. As illustrated in Fig. 48B, the exposure to dicoumarol was followed by an increase of hemoglobin release, an effect reaching statistical significance at 1 μM dicoumarol concentration. The percentage of hemolysed erythrocytes remained, however, almost one magnitude smaller than the percentage of phosphatidylserine exposing erythrocytes.

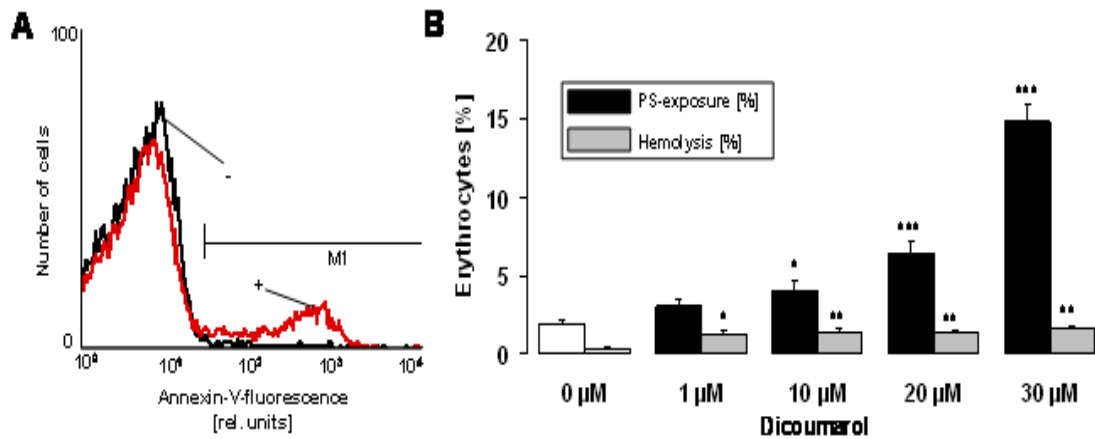


Fig. 48: Effect of dicoumarol on phosphatidylserine exposure and erythrocyte membrane integrity

A. Original histogram of annexin V binding of erythrocytes following exposure for 48 hours to Ringer's solution without (-, black line) and with (+, red line) presence of 30 μM dicoumarol.

B. Arithmetic means \pm SEM ($n = 16$) of erythrocyte annexin V binding following incubation for 48 hours in Ringer's solution without (white bar) or with (black bars) the presence of 1-30 μM dicoumarol. For comparison, arithmetic means \pm SEM ($n = 4$) of the percentage of hemolysis is shown as grey bars. *, **, *** ($p < 0.05$, $p < 0.01$, $p < 0.001$) indicate significant difference from the absence of dicoumarol (ANOVA).

Further experiments were done in the absence of extracellular Ca^{2+} to clarify, whether cell membrane scrambling depended on Ca^{2+} entry. To this end the percentage of annexin V binding erythrocytes was determined following a 48 hours treatment with dicoumarol in the presence and nominal absence of extracellular Ca^{2+} . As shown in Fig. 49, the effect of dicoumarol on annexin V-binding was virtually abolished in the nominal absence of Ca^{2+} .

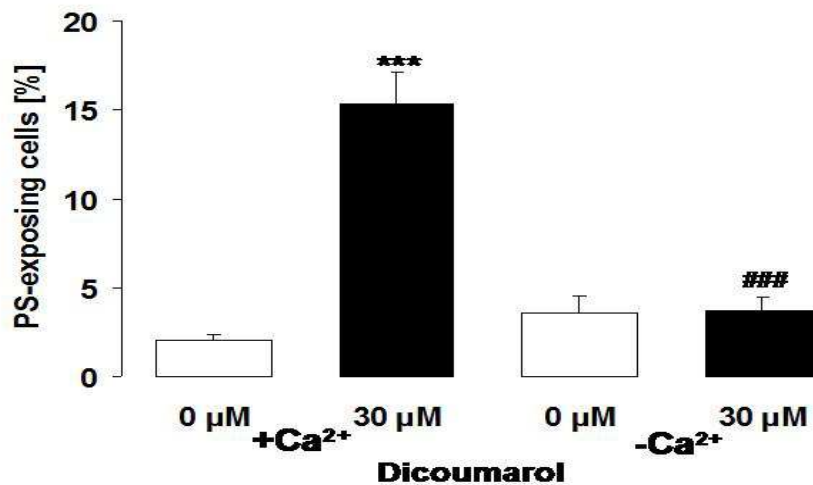


Fig. 49: Effect of Ca^{2+} withdrawal on dicoumarol induced phosphatidylserine exposure

Arithmetic means \pm SEM (n = 4) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer's solution without (white bar) or with (black bars) 30 μ M dicoumarol in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of calcium. *** (p<0.001) indicate significant difference from the absence of dicoumarol (ANOVA), ### indicates significant difference (p<0.001) from the respective values in the presence of Ca^{2+} .

8. DISCUSSION

8.1. Stimulation of suicidal death of erythrocytes by mycotoxins

8.1.1. Enniatin A

The observations from my studies reveal a novel action of the mycotoxin enniatin A, i.e. ATP depletion, triggering of Ca^{2+} entry, cell shrinkage and cell membrane scrambling in human erythrocytes. Moreover, the present observations reveal that enniatin A augments the Ca^{2+} entry and cell membrane scrambling following energy depletion. The concentrations required for those effects are well in the range of those triggering apoptosis of nucleated cells (Watjen, Debbab et al. 2009) and to be toxic *in vivo* (McKee, Bokesch et al. 1997).

As indicated earlier (Klarl, Lang et al. 2006) Ca^{2+} entry into erythrocytes is stimulated by phorbol esters and inhibited by staurosporine and chelerythrine, pointing to regulation of Ca^{2+} entry by protein kinase C (PKC). The kinase is activated and thus Ca^{2+} entry stimulated by ATP depletion (Klarl, Lang et al. 2006). The effects of energy depletion were blunted but not abolished in the presence of the kinase inhibitors staurosporine or calphostin C and mimicked by PKC stimulator phorbol-12-myristate-13-acetate or the phosphatase inhibitor okadaic acid (Klarl, Lang et al. 2006). The enniatin A-induced Ca^{2+} entry thus presumably results in large part from the severe ATP depletion. Along those lines the cell membrane scrambling was blunted by staurosporine.

An increase of cytosolic Ca^{2+} concentration is well known to trigger erythrocyte membrane scrambling (Berg, Engels et al. 2001, Bratosin, Estaquier et al. 2001). According to the present observations, enniatin A further augments the increase of cytosolic Ca^{2+} concentration following energy depletion. The increase of Ca^{2+} concentration following enniatin A exposure presumably results from stimulation of Ca^{2+} entry, which is accomplished by Ca^{2+} permeable cation channels involving TRPC6 (Foller, Kasinathan et al. 2008). Accordingly, the Ca^{2+} entry is blunted by the addition of cation channel blocker amiloride. The effect of enniatin A on cell membrane scrambling is, however, only blunted but not fully abolished in the nominal absence of extracellular Ca^{2+} indicating that the effect of enniatin A on cell membrane scrambling is in part but not completely accounted for by its effect on Ca^{2+} entry.

The effect of enniatin A is not significantly modified by the pancaspase inhibitor zVAD. This result contrasts the observations in nucleated cells, where the apoptotic effect of enniatins appears to be at least partially dependent on caspase activation (Hyun, Lee et al. 2009). Erythrocytes do express caspases (Bratosin, Estaquier et al. 2001, Mandal, Moitra et al. 2002), which could be activated by oxidative stress but do not participate in the stimulation of cell membrane scrambling by Ca^{2+} (Weil, Jacobson et al. 1998, Berg, Engels et al. 2001, Lang, Myssina et al. 2004). As illustrated in Fig. 17E, zVAD does significantly blunt the annexin V binding following exposure of erythrocytes following oxidation, indicating that the substance was able to inhibit erythrocyte caspases.

The increase of erythrocyte Ca^{2+} concentration is expected to activate Ca^{2+} sensitive K^+ channels (Bookchin, Ortiz et al. 1987, Brugnara, de Franceschi et al. 1993) leading to subsequent cell shrinkage due to exit of K^+ , hyperpolarisation of the cell membrane, exit of Cl^- and osmotically obliged water (Lang, Kaiser et al. 2003). Enniatin A exposure indeed decreased the erythrocyte forward scatter and thus led to cell shrinkage. Somewhat surprisingly, higher concentrations of enniatin A blunted the effect of glucose depletion on cell volume. Possibly, the severe ATP depletion following enniatin A treatment in the absence of glucose impaired the Na^+/K^+ ATPase activity leading to a decline of cellular K^+ concentration and thus to dissipation of the K^+ gradient across the cell membrane. Following loss of K^+ gradient across the cell membrane, the activation of Ca^{2+} sensitive K^+ channels fails to trigger K^+ exit, hyperpolarization and Cl^- exit. Thus, in K^+ depleted erythrocytes Ca^{2+} entry fails to trigger cell shrinkage. The swelling following exposure of ATP depleted erythrocytes to high (5 μM) concentrations of enniatin A could be explained by Na^+ entry through the Ca^{2+} permeable cation channels.

Similar to glucose depletion, other triggers of phospholipid scrambling could compound the effect of enniatin A. Excessive phospholipid scrambling is observed in a variety of clinical disorders (Lang, Gulbins et al. 2008) including iron deficiency (Kempe, Lang et al. 2006), phosphate depletion (Birka, Lang et al. 2004), Hemolytic Uremic Syndrome (Lang, Beringer et al. 2006), sepsis (Kempe, Akel et al. 2007), sickle cell disease (Lang, Kasinathan et al. 2009). Malaria (Foller, Bobbala et al. 2009, Lang, Kasinathan et al. 2009), Wilson's disease (Lang, Schenck et al. 2007) and possibly metabolic syndrome (Zappulla 2008). Moreover, phospholipid scrambling is triggered by a variety of further xenobiotics and endogenous substances (Mahmud, Foller et al. 2009, Bhavsar, Bobbala et al. 2010). Accelerated erythrocyte phospholipid scrambling may result in development

of anemia (Lang, Gulbins et al. 2008). Clinically overt anemia will however develop only, if the accelerated clearance of eryptotic erythrocytes cannot be compensated by increased production of new erythrocytes. The loss of circulating erythrocytes is followed by release of erythropoietin and subsequent hormonal stimulation of erythrocyte formation, which is apparent from the increase of the reticulocyte number (Foller, Feil et al. 2008, Foller, Sopjani et al. 2009). In addition to triggering anemia, erythrocyte phospholipid scrambling may impede microcirculation due to adherence of phosphatidylserine-exposing erythrocytes to the vascular wall (Wood, Gibson et al. 1996, Andrews and Low 1999). Moreover, eryptotic erythrocytes may stimulate blood clotting (Andrews and Low 1999, Zwaal, Comfurius et al. 2005, Chung, Bae et al. 2007).

In conclusion, the present observations disclose that enniatin A stimulates erythrocyte phospholipid scrambling and augments phospholipid scrambling during energy depletion. Those effects presumably contribute to the toxicity of this mycotoxin food contaminant.

8.1.2. Ochratoxin A

The present study reveals a novel effect ochratoxin A, i.e. the stimulation of erythrocyte shrinkage and plasma membrane scrambling. Thus, ochratoxin A triggers suicidal erythrocyte death or eryptosis. The concentration required for the stimulation of cell membrane scrambling is 2.5 μM , which is well in the range of the concentrations encountered *in vivo* (Alvarez, Gil et al. 2004). A plasma concentration of 2 μM , for instance, has been reported following treatment of Wistar rats with 450 $\mu\text{g}/\text{kg}$ body weight (Alvarez, Gil et al. 2004). Thus, the present observations may be relevant for the *in vivo* toxicity of ochratoxin A. On the other hand, effects of ochratoxin A in nanomolar concentrations have been described (Gekle, Sauvant et al. 2005).

Ochratoxin A is at least in part effective by increase of cytosolic Ca^{2+} -activity, which presumably results from Ca^{2+} -entry from extracellular space. Thus, ochratoxin A most likely activates Ca^{2+} -permeable cation channels. The channels have earlier been shown to involve the transient receptor potential channel TRPC6 (Foller, Kasinathan et al. 2008). The channels are known to be activated by oxidative stress (Bhavsar, Bobbala et al. 2010).

Increase of cytosolic Ca^{2+} -activity results in activation of Ca^{2+} -sensitive K^+ -channels [33, 49] leading to K^+ -exit following its chemical gradient. The resulting cell membrane hyperpolarization drives Cl^- -exit, the cellular KCl -loss is paralleled by exit of osmotically obliged water and thus by

cell shrinkage (Lang, Duranton et al. 2003). The decrease of erythrocyte volume is reflected by a decrease of forward scatter.

Increased cytosolic Ca^{2+} -activity further triggers cell membrane scrambling with subsequent phosphatidylserine exposure at the erythrocyte surface (Berg, Engels et al. 2001, Bratosin, Estaquier et al. 2001, Lang, Duranton et al. 2003). The ochratoxin A induced cell membrane scrambling is significantly blunted by removal of extracellular Ca^{2+} , indicating that ochratoxin A is at least partially effective by stimulating Ca^{2+} -entry from extracellular space. Removal of extracellular Ca^{2+} does, however, not fully abrogate the ochratoxin A induced cell membrane scrambling, indicating that one or more additional mechanisms may be involved in the stimulation of cell membrane scrambling by ochratoxin A.

Such an additional mechanism contributing to the ochratoxin A induced cell membrane scrambling is the stimulation of ceramide formation. Ceramide sensitizes the scrambling machinery for Ca^{2+} and may by itself stimulate cell membrane scrambling in erythrocytes (Lang, Myssina et al. 2004, Lang, Gulbins et al. 2010). Moreover, ceramide is a powerful stimulator of apoptosis (Kornhuber, Tripal et al. 2010). Ceramide participates in the pathophysiology of several diseases, including diabetes (Zhao, Przybylska et al. 2007, Holland and Summers 2008), Wilson's disease (Lang, Schenck et al. 2007), multiple sclerosis (Walter and Fassbender 2010), major depression (Kornhuber, Tripal et al. 2010), Parkinson's disease (Arboleda, Huang et al. 2007), Alzheimer's disease (de la Monte 2009, Tong, Neusner et al. 2009, Kornhuber, Tripal et al. 2010), cardiovascular disease (Kuebler, Yang et al. 2010, Li, Becker et al. 2010), cystic fibrosis (Becker, Grassme et al. 2010), lung inflammation, fibrosis and infection (Dhami, He et al. 2010). However, inhibition of sphingomyelinase with amitriptylin did not prevent the stimulation of cell membrane scrambling by ochratoxin A. Thus, activation of the sphingomyelinase may contribute to but does not account for the stimulation of cell membrane scrambling by ochratoxin A.

Eryptosis may be pathophysiologically relevant. Phosphatidylserine exposing erythrocytes adhere to endothelial CXCL16/SR-PSO (Borst, Abed et al. 2012). The erythrocyte adhesion to the vascular wall is expected to compromise microcirculation (Wood, Gibson et al. 1996, Borst, Abed et al. 2012). Phosphatidylserine exposing erythrocytes are further known to foster blood clotting (Andrews and Low 1999, Zwaal, Comfurius et al. 2005, Chung, Bae et al. 2007) and thus to increase the risk of thrombosis. Eventually, phosphatidylserine exposing erythrocytes are engulfed and thus cleared from circulating blood (Lang, Gulbins et al. 2008). To the extent that

the accelerated loss of erythrocytes is not compensated by a similar increase of erythropoiesis, enhanced eryptosis may result in anemia (Lang, Gulbins et al. 2008). Thus, the present observations could well explain the ochratoxin induced anemia known since several decades (Huff, Chang et al. 1979, Stoev, Anguelov et al. 2000).

The present observations may be relevant not only for the ochratoxin A induced erythrocyte death, but may, in addition, shed additional light on the mechanisms underlying ochratoxin A induced apoptosis of nucleated cells. Evidence for the involvement of Ca^{2+} in the stimulation of apoptosis has been provided before [29]. To the best of our knowledge, the involvement of ceramide formation in ochratoxin A toxicity has never been reported. In view of the present observations, ceramide may be involved in ochratoxin A induced apoptosis of nucleated cells.

In conclusion, the exposure of erythrocytes to ochratoxin A leads to Ca^{2+} entry and ceramide formation with subsequent triggering of cell membrane scrambling and cell shrinkage. Thus, ochratoxin A stimulates eryptosis, the suicidal death of erythrocytes.

8.1.3. Zearalenone

The present observations uncover a novel effect of zearalenone, i.e. triggering of eryptosis, the suicidal death of erythrocytes. Exposure of human erythrocytes to zearalenone triggered the two hallmarks of eryptosis, i.e. erythrocyte shrinkage and erythrocyte membrane scrambling. The concentrations required were one order of magnitude higher (10-25 μM) than the concentrations found in blood of the common population (Pillay, Chuturgoon et al. 2002). The recommended safe human intake is estimated to be 0.05 $\mu\text{g}/\text{kg}$ of body weight per day (Bennett and Klich 2003). At least in theory the concentration required for eryptosis may be reached by individuals exposed to cereals with excessive mycotoxin content.

Zearalenone exposure decreased cell volume most likely by stimulating Ca^{2+} entry with subsequent increase of cytosolic Ca^{2+} activity. The increased cytosolic Ca^{2+} concentration activates Ca^{2+} sensitive K^+ channels (Bookchin, Ortiz et al. 1987, Brugnara, de Franceschi et al. 1993) leading to cell membrane hyperpolarization, potential driven Cl^- exit and thus cellular loss of KCl together with osmotically obliged water (Lang, Kaiser et al. 2003).

The increase of cytosolic Ca^{2+} activity further accounted in large part for the stimulation of cell membrane scrambling. Accordingly, zearalenone induced cell membrane scrambling was virtually abrogated in the absence of extracellular Ca^{2+} . Most xenobiotics known to trigger eryptosis are

effective in large part by increasing cytosolic Ca^{2+} activity (Lang, Gulbins et al. 2008). Circumstantial evidence suggests that Ca^{2+} enters into erythrocytes in part through Ca^{2+} permeable non-selective cation channels involving the transient receptor potential channel TRPC6 (Foller, Kasinathan et al. 2008). The Ca^{2+} permeable erythrocyte cation channels include oxidative stress (Brand, Sandu et al. 2003). Zearalenone is known to induce oxidative stress (Bouaziz, Sharaf El Dein et al. 2008, Bouaziz, Martel et al. 2009, Banjerdpongchai, Kongtawelert et al. 2010, Salah-Abbes, Abbes et al. 2010, Yu, Zheng et al. 2011) and to increase cytosolic Ca^{2+} concentration (Vlata, Porichis et al. 2006) in nucleated cells.

Circulating eryptotic erythrocytes are rapidly cleared from from blood (Lang, Gulbins et al. 2008). Thus, even after stimulation of eryptosis, the percentage of eryptotic erythrocytes remains low *in vivo*. The accelerated loss of eryptotic erythrocytes following *in vivo* stimulation of erythrocytes may lead to anemia (Lang, Gulbins et al. 2008). Moderate stimulation of eryptosis may, however, be fully compensated by parallel stimulation of erythrocyte formation (Lang, Gulbins et al. 2008). Anemia develops as soon as the rate of eryptosis exceeds the formation of new erythrocytes, (Lang, Gulbins et al. 2008).

Phosphatidylserine exposing erythrocytes may adhere to endothelial CXCL16/SR-PSO (Borst, Abed et al. 2012) and thus attach to the vascular wall. Moreover, phosphatidylserine exposing erythrocytes may trigger blood clotting and thus lead to thrombosis (Andrews and Low 1999, Zwaal, Comfurius et al. 2005, Chung, Bae et al. 2007). Phosphatidylserine exposing erythrocytes may thus compromise microcirculation (Wood, Gibson et al. 1996, Andrews and Low 1999, Closse, Dachary-Prigent et al. 1999, Gallagher, Chang et al. 2003, Pandolfi, Di Pietro et al. 2007, Borst, Abed et al. 2012).

In conclusion, exposure of human erythrocytes to zearalenone stimulates Ca^{2+} entry, leading to eryptosis, the suicidal erythrocyte death characterized by cell membrane scrambling and cell shrinkage.

8.2. Stimulation of suicidal death of erythrocytes by phytochemicals

8.2.1. Withaferin A

According to the present observations withaferin A triggers eryptosis, the suicidal death of erythrocytes. Withaferin A exposure leads to the two hallmarks of eryptosis. i.e. erythrocyte membrane scrambling and erythrocyte shrinkage. The concentration required for the effect on cell membrane scrambling is in the range of the concentrations effective against tumor cells (Samadi, Tong et al. 2010) and is only slightly above the concentrations observed *in vivo* after therapeutic administration of withaferin A (Thaiparambil, Bender et al. 2011).

Withaferin A increases cytosolic Ca^{2+} activity, an effect pointing to activation of Ca^{2+} permeable cation channels. Erythrocytes do express Ca^{2+} permeable unselective cation channels (Lang, Gulbins et al. 2008), which are in part dependent on the expression of transient receptor potential channel TRPC6 (Foller, Kasinathan et al. 2008). The cation channels have previously been shown to be activated by oxidative stress (Lee, Um et al. 2009, Hahm, Moura et al. 2011, Mayola, Gallerne et al. 2011, Yang, Choi et al. 2011). According to 2',7' dichlorodihydrofluorescein diacetate (DCFDA) fluorescence, withaferin A indeed induces oxidative stress which could well account for activation of the channels.

The increase of cytosolic Ca^{2+} activity presumably contributes to or even accounts for the observed cell shrinkage. Erythrocytes do express Ca^{2+} sensitive K^+ channels (Lang et al., 2008). Activation of those channels leads to exit of K^+ following its chemical gradient which in turn leads to cell membrane hyperpolarisation and potential driven Cl^- exit. The cellular loss of KCl with osmotically obliged water leads to cell shrinkage (Lang, Gulbins et al. 2008), which is reflected by the observed decrease of forward scatter.

An increase of cytosolic Ca^{2+} activity further triggers cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface (Lang, Gulbins et al. 2008). The cell membrane scrambling following treatment with withaferin A is blunted, but not abrogated by removal of extracellular Ca^{2+} . Thus, Ca^{2+} entry contributes to but does not fully account for the triggering of the cell membrane scrambling following withaferin treatment and withaferin A thus induces cell membrane scrambling by some additional mechanism(s).

Withaferin A treatment was further followed by an increase in the formation of ceramide, which has previously been shown to stimulate erythrocyte membrane scrambling (Lang, Gulbins et al. 2010). Ceramide similarly triggers apoptosis of nucleated cells (Kornhuber, Tripal et al. 2010) and ceramide formation participates in the pathophysiology of several clinical disorders including lung inflammation, fibrosis and infection (Dhami, He et al. 2010), cystic fibrosis (Becker, Grassme et al. 2010), cardiovascular disease (Kuebler, Yang et al. 2010, Li, Becker et al. 2010), Wilson's disease (Lang, Schenck et al. 2007), multiple sclerosis (Walter and Fassbender 2010), major depression (Kornhuber, Tripal et al. 2010), Parkinson's disease (Arboleda, Huang et al. 2007), Alzheimer's disease (de la Monte 2009, Tong, Neusner et al. 2009, Kornhuber, Tripal et al. 2010) and diabetes (Zhao, Przybylska et al. 2007, Holland and Summers 2008, Zigmond, Zangen et al. 2009). Thus, withaferin induced ceramide formation may affect survival and function of a wide variety of cells.

Phosphatidylserine exposing erythrocytes are engulfed by macrophages (Lang, Gulbins et al. 2008) and are thus eventually cleared from circulating blood (Lang, Gulbins et al. 2008). Accordingly, excessive eryptosis leads to anemia (Lang, Gulbins et al. 2008). Phosphatidylserine exposing erythrocytes further adhere to endothelial CXCL16/SR-PSOX (Borst, Abed et al. 2012). The adherence of erythrocytes to the vascular wall may impair microcirculation (Borst, Abed et al. 2012). Moreover, phosphatidylserine exposing erythrocytes foster blood clotting (Zwaal, Comfurius et al. 2005, Chung, Bae et al. 2007). Accordingly, excessive eryptosis may result in thrombosis.

In conclusion, withaferin A triggers Ca^{2+} entry, oxidative stress and ceramide formation, effects triggering eryptosis, which is characterized by cell membrane scrambling and cell shrinkage. Thus, withaferin A triggers not only apoptosis in nucleated cells with mitochondria, but is similarly effective in cells lacking those important organelles in the machinery of apoptosis.

8.2.2. Oridonin

The present observations unravel a completely novel effect of oridonin, i.e. increase of cytosolic Ca^{2+} activity and stimulation of ceramide formation in erythrocytes, events eventually leading to eryptosis, the suicidal death of erythrocyte. Oridonin has a minor stimulating effect on hemolysis.

Oridonin induced annexin V binding is blunted in the nominal absence of Ca^{2+} , indicating that the cell membrane scrambling is in part secondary to the increase of cytosolic Ca^{2+} activity. According

to earlier observations (Foller, Kasinathan et al. 2008), Ca^{2+} enters the erythrocytes through Ca^{2+} permeable cation channels involving the transient receptor potential channel TRPC6. To the best of our knowledge, an effect of oridonin on ion channels or on cytosolic Ca^{2+} activity has never been reported.

The increase of erythrocyte Ca^{2+} concentrations activates Ca^{2+} sensitive K^+ channels (Bookchin, Ortiz et al. 1987, Brugnara, de Franceschi et al. 1993) with subsequent K^+ exit, hyperpolarisation of the cell membrane, Cl^- exit, and cellular loss of KCl with osmotically obliged water (Lang, Kaiser et al. 2003). Oridonin thus decreases the forward scatter.

The effect of oridonin on cell membrane is not completely abrogated in the nominal absence of extracellular Ca^{2+} . Thus, oridonin must have an additional effect on erythrocytes. As a matter of fact, oridonin stimulates the formation of ceramide, which has previously been shown to participate in the triggering of apoptosis and/or cell membrane scrambling in a variety of cells (Arana, Gangoiti et al. 2010, Nikolova-Karakashian and Rozenova 2010) including neurons (Jana, Hogan et al. 2009, Arboleda, Cardenas et al. 2010), T-lymphocytes (Gulbins, Szabo et al. 1997), hepatocytes (Lang, Schenck et al. 2007), epithelial cells (Teichgraber, Ulrich et al. 2008) and erythrocytes (Lang, Myssina et al. 2004, Lang, Schenck et al. 2007, Lang, Gulbins et al. 2010). Oridonin does not induce energy depletion, another potential stimulator of eryptosis (Klarl, Lang et al. 2006).

In nucleated cells, oridonin triggers apoptosis in part by affecting the mitochondria (Zhang, Wu et al. 2004, Li, Cui et al. 2007, Huang, Wu et al. 2008, Li, Wu et al. 2008). Erythrocytes are, however, devoid of mitochondria and the stimulation of Ca^{2+} entry and subsequent triggering of cell shrinkage as well as cell membrane scrambling do not require prior depolarization of the mitochondrial membrane.

In nucleated cells, oridonin is further partially effective through inhibition of the transcription factor NF κ B (Hsieh, Wijeratne et al. 2005, Ikezoe, Yang et al. 2005, Cho, Moon et al. 2009, Zhang, Wu et al. 2009, Gatsinzi and Iverfeldt 2011, Ma, Xu et al. 2011) As erythrocytes lack nuclei, the observed eryptosis cannot be secondary to altered gene expression. It is noteworthy, though, that NF κ B inhibitors Bay 11-7082 and Parthenolide have most recently been shown to trigger eryptosis (Ghashghaieina, Toulany et al. 2011). Whether the effect of the inhibitors is indeed caused by NF κ B inhibition, remained, however, elusive.

Oridonin may augment the eryptosis resulting from other causes (Lang, Gulbins et al. 2008) such as iron deficiency (Kempe, Lang et al. 2006), phosphate depletion (Birka, Lang et al. 2004), sepsis (Kempe, Akel et al. 2007), Hemolytic Uremic Syndrome (Lang, Beringer et al. 2006), sickle cell disease (Lang, Kasinathan et al. 2009) malaria (Lang, Schenck et al. 2007, Koka, Bobbala et al. 2009, Siraskar, Ballal et al. 2010), Wilson's disease (Lang, Schenck et al. 2007) and presumably metabolic syndrome (Zappulla 2008). Moreover, oridonin may potentiate the eryptotic effect of other xenobiotics (Mahmud, Mauro et al. 2009, Bhavsar, Bobbala et al. 2010).

Eryptosis may result in anemia (Lang, Gulbins et al. 2008) and adherence of phosphatidylserine-exposing erythrocytes to the vascular wall may compromise microcirculation (Wood, Gibson et al. 1996, Andrews and Low 1999). Eryptotic erythrocytes may further trigger blood clotting (Andrews and Low 1999, Zwaal, Comfurius et al. 2005, Chung, Bae et al. 2007). In conclusion, the present study reveals a completely novel effect of oridonin, i.e. increase of cytosolic Ca^{2+} activity and stimulation of ceramide formation in erythrocytes, effects eventually triggering eryptosis, the suicidal erythrocyte death.

8.2.3. Dicoumarol

The present study reveals a novel side effect of dicoumarol, i.e. activation of the erythrocyte cation channel with subsequent triggering of Ca^{2+} entry into and eryptosis of human erythrocytes. The dicoumarol concentrations required for those effects are in the range of those approached *in vivo*. Plasma concentrations of dicoumarol have been reported to approach $7.7 \pm 1.1 \mu\text{g/ml}$ or approximately $20 \mu\text{M}$ (Levy, Lai et al. 1978).

The eryptosis is apparently the result of Ca^{2+} entry with subsequent increase of the cytosolic Ca^{2+} concentration. At higher dicoumarol concentrations activation of current and Ca^{2+} entry are observed within a few hours and could thus account for the triggering of cell membrane scrambling within 48 hours. Accordingly, the phosphatidylserine exposure is abrogated in the absence of extracellular Ca^{2+} . The Ca^{2+} entry into erythrocytes involves TRPC6 (Foller, Kasinathan et al. 2008). Dicoumarol may stimulate the channels indirectly. For instance, dicoumarol may induce oxidative stress (Collier and Pritsos 2003, Jing, Yi et al. 2004, Vad, Yount et al. 2009), which in turn activates the erythrocyte cation channels (Duranton, Huber et al. 2002). On the other hand, dicoumarol may counteract oxidation (Mangelus, Kroyter et al. 2001, Hasegawa, Bando et al. 2004). Thus, the mechanism of channel activation remains elusive. Whatever mechanism

involved in channel activation, the increase of cytosolic Ca^{2+} concentration triggers erythrocyte membrane scrambling with subsequent phosphatidylserine exposure at the cell surface (Berg, Engels et al. 2001, Bratosin, Estaquier et al. 2001). At higher concentrations (30 μM), dicoumarol may, in addition, stimulate the formation of ceramide (data not shown), which sensitizes the cell membrane for the cell membrane scrambling effect of cytosolic Ca^{2+} (Lang, Myssina et al. 2004, Lang, Gulbins et al. 2010).

Cytosolic Ca^{2+} further activates Ca^{2+} sensitive K^+ channels (Bookchin, Ortiz et al. 1987, Brugnara, de Franceschi et al. 1993) with subsequent exit of K^+ , hyperpolarisation of the cell membrane, exit of Cl^- and thus cellular loss of KCl with osmotically obliged water (Lang, Kaiser et al. 2003). Along those lines, exposure of erythrocytes to dicoumarol decreased the erythrocyte forward scatter, an observation pointing to cell shrinkage.

Dicoumarol may exacerbate eryptosis triggered in the course of several clinical conditions (Lang, Gulbins et al. 2008) including iron deficiency (Kempe, Lang et al. 2006), phosphate depletion (Birka, Lang et al. 2004), Hemolytic Uremic Syndrome (Lang, Beringer et al. 2006), sepsis (Kempe, Akel et al. 2007), sickle cell disease (Lang, Kasinathan et al. 2009). Malaria (Foller, Bobbala et al. 2009, Koka, Bobbala et al. 2009, Lang, Kasinathan et al. 2009, Bobbala, Alesutan et al. 2010, Siraskar, Ballal et al. 2010), Wilson's disease (Lang, Schenck et al. 2007) and possibly metabolic syndrome (Zappulla 2008). Eryptosis is further stimulated by a wide variety of small molecules (Braun, Foller et al. 2009, Gatidis, Foller et al. 2009, Mahmud, Foller et al. 2009, Bhavsar, Bobbala et al. 2010, Bhavsar, Eberhard et al. 2010, Eberhard, Ferlinz et al. 2010). The pro-eryptotic effect of dicoumarol could be compounded in those clinical conditions and in the presence of those chemicals.

Erythrocyte phospholipid scrambling fosters the clearance of affected erythrocytes from circulating blood (Lang, Gulbins et al. 2008). To the extent that the loss of erythrocytes cannot be compensated by release of erythropoietin and subsequent hormonal stimulation of erythrocyte formation (Foller, Feil et al. 2008, Foller, Sopjani et al. 2009) the loss of erythrocytes may lead to overt anemia. Moreover, erythrocyte phospholipid scrambling may foster adherence of phosphatidylserine-exposing erythrocytes to the vascular wall and thus impede microcirculation (Wood, Gibson et al. 1996, Andrews and Low 1999). Eryptotic erythrocytes may further stimulate blood clotting (Andrews and Low 1999, Zwaal, Comfurius et al. 2005, Chung, Bae et al. 2007).

At least in theory, dicoumarol may similarly stimulate Ca^{2+} entry into nucleated cells, which may trigger of apoptosis (Berridge, Lipp et al. 2000, Svoboda, Pruetting et al. 2009). Moreover, apoptosis may be triggered by ceramide (Arboleda, Huang et al. 2007, Lang, Schenck et al. 2007, Teichgraber, Ulrich et al. 2008) . Thus, the ability of dicoumarol to stimulate Ca^{2+} entry and generate ceramide formation may result in apoptosis.

In conclusion, the present observations disclose that dicoumarol exposure leads to cell shrinkage and cell membrane scrambling, effects largely due to Ca^{2+} entry.

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