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The effect of camalexin and calyculin A on eryptosis

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List of Abbreviations

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CNS	Central nervous system
COX	Cyclooxygenase
Ctr	Control
EDTA	Ethylenediaminetetraacetic acid
EGTA	Glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
EPO	Erythropoietin
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FasR	Fas receptor
FITC	Fluorescein isothiocyanate
FL	Fluorescence channel
FMK	Fluoromethyl ketone
FSC	Forward scatter
G	gram
GLUT	Glucose transporter
GSH	Glutathione
H	hour
HB	Hemoglobin
HCT	Hematocrit
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid
IL	Interleukin
IONO	Ionomycin
JAK	Janus kinase
K+	Potassium
KCC	K^+/Cl^- cotransporter
mM	Millimolar
Na^+	Sodium
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NHE	Na^+/H^+ exchanger
NO	Nitric oxide
PAF	Platelet activating factor
PBS	Phosphate-buffered saline
PGE2	Prostaglandin E2
PGN	Peptidoglycan
PLA	Phospholipase A
PPAR	Peroxisome proliferator-activated receptor
PP1/2A	Protein phosphatase 1/2A

PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SM	Sphingomyelin
SSC	Side scatter
T	Time
μM	Micromolar
z.B	zum Beispiel

1. INTRODUCTION

1.1. Apoptosis

The term apoptosis was coined in 1972 by Kerr, Wyllie, and Currie **(Kerr et al., 1972)**. In Greek, the term apoptosis means “to fall away from”. Apoptosis, or programmed cell death, is an irreversible process of cellular suicide, characterized by specific morphological changes, and is mediated by energy-based biochemical mechanisms **(Elmore, 2007)**. During this process, cellular fragments called apoptotic bodies “fall away from” cells as they break down. The clearance of apoptotic bodies occurs by phagocytosis without an inflammatory response **(Gulbins et al., 2000)**. When erythrocytes undergo apoptosis, this process is termed eryptosis **(Kerr, 2002)**.

Physiologically, apoptosis is recognized as a homeostatic mechanism that occurs during development and aging, mainly maintaining cell populations in tissues **(Renehan et al., 2001)**. Furthermore, it acts as a host defense mechanism **(Norbury and Hickson, 2001)**. Typical morphological features of apoptotic cells are shrinkage, membrane blebbing, nuclear and DNA fragmentation, and chromatin condensation. Following the occurrence of these morphological changes, apoptotic cells are engulfed by macrophages **(Kurosaka et al., 2003) (Savill and Fadok, 2000) (Fadok et al., 2000)**.

Proteases called caspases play an important role in the breakdown of cellular structures during apoptosis. Caspases are normally inactive, and are activated when cells receive apoptotic signals. This activation can occur via two major pathways in mammalian cells: The extrinsic pathway, also known as the death receptor pathway, and the intrinsic pathway. Both pathways occur separately and then converge into a single pathway during the final stages of apoptosis **(Igney and Krammer, 2002)**. The extrinsic pathway is stimulated by various triggers involving members of the TNF cytokine receptor family and their corresponding substrates, including the ligand/receptor pairs TNF-α/TNFR1, FasL/FasR, Apo2L/DR5, Apo3L/DR3, and Apo2L/ DR4 **(Suliman et al., 2001) (Rubio-Moscardo et al., 2005)**. These proteins mediate adapter proteins to their cytosolic death domains (DDs), including Fad, which then binds death effector domain-containing (DED-containing) procaspases, particularly procaspase-8, resulting in their activation.

On the other hand, the intrinsic mechanism, also known as the mitochondrial pathway,

depends on mitochondrial heme proteins such as cytochrome c, which are released into the cytosol. This release occurs as a result of several events, including non-receptor stimuli or an increase in the level of the pore-forming pro-apoptotic Bcl-2 family of proteins, such as Bax. These intracellular events act directly on the mitochondria without any intermediates, leading to changes in the inner mitochondrial membrane. Accordingly, a process called mitochondrial permeability transition (MPT) occurs, leading to pore opening, loss of transmembrane potential, and the release of two groups of intermembrane pro-apoptotic proteins **(Festjens et al., 2004)**. The protein groups comprise cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi **(Garrido et al., 2006**). In the cytosol, cytochrome c binds to Apaf-1, forming a complex that leads to the activation of procaspase-9. The active forms of caspase-9 (intrinsic) and caspase-8 (extrinsic) have been shown to cleave and activate the effector protease caspase-3 directly. The second group comprises apoptosis-inducing factor (AIF), endonuclease G, and CAD, which are released by the mitochondria during the final stage of apoptosis. AIF causes DNA fragmentation and chromatin condensation **(Joza, et al., 2001)**. The Bcl-2 protein family regulates all of these mitochondrial apoptotic events **(Cory and Adams, 2002)**.

In contrast, the other major type of cell death, necrosis, occurs in response to cellular trauma or acute injury as a result of various factors including chemical, physical (thermal, mechanical, electric, radiations), microbiological (bacterial, viral, fungal, protozoa), immunological, genetic, ischemic, and endocrine factors, as well as nutritional imbalances **(Hawkins et al, 1972; Alison and Sarraf, 1994; Rosette et al., 1996 Bachmeier et al., 2007)**.

Although both apoptosis and necrosis lead to cell death, there are several striking differences. Whereas controlled cell shrinkage, membrane blebbing, and the regulated breakdown of nuclear genetic and mitochondrial material are hallmarks of apoptotic cells, cell death in necrotic cells is dramatic and unregulated **(Wyllie et al, 1980)**. Necrotic cells may form vacuoles on their surfaces or swell with internal structural changes, rapid distention, resulting in the destruction of cellular processes and chemical structures **(Chan, 2013)**.

1.2. Eryptosis

Erythrocyte senescence with subsequent erythrocyte death plays a major role in the clearance of aged red blood cells **(Kiefer et al., 2000)**. As there are some cellular structural similarities as well as differences with apoptosis, the term eryptosis is preferably used to represent the suicidal death of erythrocytes. Eryptosis prevents the hemolysis of erythrocytes and, therefore, its negative impact, such as the filtration and precipitation of free hemoglobin through the renal glomeruli and tubules, leading to renal failure **(Lang & Lang, 2015b) (Lang & Föller, 2012c)**.

Phosphatidylserine exposure at the outer cell surface triggers macrophages, which possess specific receptors that detect the phosphatidylserine exposed on the erythrocyte surface during apoptosis. This leads to the degradation of the engulfed erythrocytes and their clearance from the circulation **(Boas, Forman et al. 1998)**.

In the process of eryptosis, aged or impaired erythrocytes pass through several stages, with cells destined for programmed death characterized by cellular diminution, membrane blebbing, protease activation, and phosphatidylserine exposure at the cell surface **(Lang & Lang, 2015b) (Lang & Föller, 2012c)**, events similar to apoptosis of nucleated cells **(Montague et al., 1999)**. Phosphatidylserine exposure at the outer cell surface stimulates macrophages, which possess specific receptors that detect the phosphatidylserine exposed on the erythrocyte surface during apoptosis. This leads to the degradation of the engulfed erythrocytes and their clearance from circulating blood.

Eryptosis, therefore, controls the lifespan of erythrocytes. Under normal physiological conditions, the average lifespan of an erythrocyte is limited to 100–120 days **(Lew and Bookchin, 2005**) **(Bosman et al., 2005)**. However, in many clinical disorders, where eryptosis is pathologically deregulated in response to stressors, erythrocyte lifespan is significantly reduced **(Lang et al., 2002)**.

These stressors include oxidative or osmotic stress, which ultimately result in caspase activation **(Bratosin et al., 2001) (Mandal et al., 2003)**. Eryptosis primarily represents a mechanism of defective erythrocytes avoiding hemolysis in response to the abovementioned stressors. Therefore, the lack of restraint on eryptosis leads to the development of anemia **(Lang, Gulbins et al. 2008)**, highlighting the importance of its

regulation. Conditions with severe eryptosis include iron deficiency, lead or mercury poisoning, malaria **(Föller & Lang, 2008)**, sickle cell anemia, G6PD deficiency, and thalassemia **(Lang et al., 2002)**. Additionally, infection with hemolysin-forming pathogens results in a lack of restraint and enhances eryptosis **(Lang, et al., 2004)**.

To prevent uncontrolled eryptosis, this process is inhibited by erythropoietin (EPO), which inhibits the Ca^{2+}permeable cation channels **(Kiefer & Snyder, 2000) (Myssina et al., 2003b)**. Thus, EPO extends the average length of time of erythrocytes circulating in the blood **(Polenakovic et al., 1996) (Lang & Wieder, 2006)**.

1.3. Eryptotic inducers

Numerous clinical conditions, as well as a broad range of endogenous mediators and xenobiotics, have been implicated in eryptosis. Various inducing procedures, such as osmotic shock **(Lang et al., 2003)**, oxidative stress **(Duranton, Huber et al. 2002)** and ATP depletion **(Lang et al., 2002),** result from a group of clinical conditions. These include beta-thalassemia **(Lang et al., 2002)**, chronic kidney disease (CKD) **(Abed, et al., 2014),** diabetes **(Nicolay & Lang 2006)**, hemolytic-uremic syndrome **(Lang & Lang, 2015a),** sepsis **(Kempe et al., 2007)**, sickle cell disease **(Lang & Lang, 2015a)**, Wilson disease **(Lang & Lang, 2007)**, hemoglobin C (HbC) deficiency, G6PD deficiency **(Lang et al., 2002)**, and malignancies, which have been reported to be promoters of eryptosis **(Lang et al., 2012b).** Potential xenobiotic stimulators of eryptosis are aluminum **(Niemoeller et al., 2006b)**, gold **(Sopjani et al., 2008c)**, selenium **(Sopjani et al., 2008b)**, cadmium **(Sopjani et al., 2008a),** lead **(Kempe et al., 2005)**, tin **(Nguyen et al., 2009)**, zinc **(Kiedasch et al., 2008)**, amiodarone **(Nicolay et al., 2007)**, amphotericin b **(Mahmud et al., 2009),** chlorpromazine **(Koka et al., 2008b)**, cyclosporine **(Niemoeller et al., 2006a)**, hemolysin **(Lang, et al., 2004)**, methyldopa **(Mahmud et al., 2008),** and valinomycin **(Lang et al., 2012a; Lupescu et al., 2012)**. The majority of xenobiotics induce eryptosis by enhancing cytosolic Ca^{2+}activity and prostaglandin E2 **(Lang et al., 2005c) (Lang & Lang, 2005b)**. Eryptosis could also be augmented by stimulating ceramide formation by methyldopa and menadione **(Qadri et al., 2009b)**. There are also definite triggers of eryptosis, such as phytic acid, which only lower the cellular ATP

content **(Eberhard et al., 2010)**. Eryptosis induced by thymoquinone occurs via initiation of the protein kinase C pathway without appreciable stimulation of ceramide or intracellular calcium activity **(Qadri et al., 2009a)**.

1.4. Eryptotic inhibitors

Eryptosis can also be inhibited by various factors such as nitric oxide **(Nicolay et al., 2008)** and catecholamine **(Lang et al., 2005a)**. EPO may partially inhibit the Ca^{2+} permeable cation channels thus counteracting eryptosis **(Myssina et al., 2003)**.

Eryptosis is also inhibited by resveratrol **(Qadri et al., 2009c)**, caffeine **(Floride et al., 2008)**, zidovudine **(Kucherenko et al., 2008)**, and adenosine **(Niemoeller et al., 2007)**. In addition, staurosporine can impede eryptosis by inhibiting protein kinase C **(Klarl, et al., 2006)**. The suicidal impact on erythrocytes can also be attenuated by urea, which abolishes ceramide formation **(Lang, et al., 2004)**. The inhibition of PGE2 formation and its antioxidant activity caused the inhibition of Ca^{2+} permeable cation channels, resulting in the attenuation of eryptosis **(Lang & Lang, 2005b)**.

1.5. Molecular mechanisms of eryptosis

The molecular mechanisms that underlie the initiation of eryptosis have been elucidated in detail. Two different signal transduction pathways collaborate to initiate eryptosis. In the first process, prostaglandin E2 (PGE2) is formed, causing Ca^{2+} permeable cation channel opening **(Lang & Lang, 2005b)**, and the resulting increase in intracellular Ca^{2+}. In the second process, phospholipase A2 mediates the release of platelet activating factor (PAF), which, in turn, activates sphingomyelinase, causing the production of ceramide **(Lang et al., 2005c)**.

Together, increased Ca^{2+} and ceramide levels cause membrane scrambling, which culminates in phosphatidylserine exposure at the cell surface to signal phagocytosis **(Berg et al., 2001) (Lang & Lang, 2015b)**. Furthermore, Ca^{2+} initiates the stimulation of calcium-sensitive potassium channels, leading to a marked reduction of KCl concentration in the cells, resulting in significant cellular volume decrease **(Lang &**

Lang, 2015b). In addition, the protease calpain is activated by calcium, leading to the breakdown of the cytoskeleton and fragmentation breakdown **(Bratosin et al., 2001)** (**Lang et al., 2005d) (Lang et al., 2007)**. The various components of these pathways are discussed in further detail below.

1.5.1. Ca^{2+} permeable non-selective cation channels

Activation of monovalent and divalent cation-permeable channels, known as non-selective cation channels, results in a marked rise in cytosolic Ca^{2+} activity in eryptosis **(Kaestner et al., 2002) (Kaestner et al., 2004)**. Currently, cation channels are known to comprise the TRPC6 channel, a transient receptor potential channel. Cation channels can be activated by osmotic shock, oxidative stress **(Lang et al., 2003) (Duranton et al., 2002)**, and Cl^- removal **(Duranton et al., 2002) (Huber, Gamper, & Lang, 2001) (Lang et al., 2005d)**. The channels are activated by stimulation of cyclooxygenase (COX) with subsequent PGE2 synthesis **(Lang & Lang, 2005b)**.

Increased intracellular Ca^{2+} augments Gardos channel (Ca^{2+} sensitive K^+ channel) activation, mediating K^+ loss as well as Cl^- loss via parallel anion pathways, resulting in osmotic water loss and subsequently cell shrinkage. The high intracellular Ca^{2+} concentration triggers the enzyme scramblase, which increases the exposure of phosphatidylserine in the outer surface of the erythrocyte membrane **(Bratosin et al., 2001) (Maher and Kuchel, 2003) (Lang et al., 2007)**.

1.5.2. Cl^- channels

In general, Cl^- channels play a vital role in controlling the ionic composition of the cytoplasm and cell volume **(Kucherenko et al., 2013)**. Furthermore, they play a fundamental role in eryptosis, with their activation leading to cell membrane depolarization through Cl^- efflux, which usually occurs concurrently with the exit of K^+. Osmotic water loss and successive cell shrinkage occur because of the loss of osmotically active KCl, which is considered one of the most well-known consequences of eryptosis **(Lang et al., 2003)**.

1.5.3. Mechanism of oxidative stress in eryptosis

Oxidative stress is one of the most common triggers of eryptosis **(Han et al., 2004) (Varela et al., 2007)**. Defects of anti-oxidative defense partially initiate eryptosis by triggering the influx of Ca^{2+} ion entry via the onset and stimulation of the Ca^{2+} permeable cation channels. In addition, oxidative stress activates erythrocyte Cl^- channels. Stimulation of eryptosis by oxidative stress has been shown to occur simultaneously with aspartyl and cystinyl proteases activation **(Lang et al., 2006)**. Specific caspases have been identified to be manifested markedly inside erythrocytes and activated in response to oxidative stress. Furthermore, caspases share in the splitting and cracking of the anion exchanger band and stimulating phosphatidylserine exposure **(Berg et al., 2001)**.

1.5.4. Cation channel activation via PGE2 formation and COX activation

Two signaling pathways initiate eryptosis. Prostaglandin E2 formation results in Ca^{2+} permeable cation channel activation in response to Cl^- ion removal and hyperosmotic shock. In turn, intracellular Ca^{2+} levels increase, stimulating membrane vesiculation followed by phosphatidylserine exposure on the erythrocyte outer membrane **(Lang & Lang, 2012)**.

The role of PGE2 has been proven via the activation of the Ca^{2+}-dependent cysteine endopeptidase calpain, which is nonessential for phosphatidylserine exposure on the outer leaflet. However, calpain has been demonstrated to mediate cytoskeletal fragmentation and breakdown **(Bratosin et al., 2001)** (**Lang et al., 2005d) (Lang et al., 2007)**. Energy depletion activates the cation channels that weaken the antioxidative defense of erythrocytes via the replenishment of glutathione (GSH) **(Lang et al., 2006)**.

1.5.5. PAF synthesis and sphingomyelinase activation

Erythrocyte volume compression further initiates the generation of PAF, which plays a role in sphingomyelinase activation, resulting in the breakdown of sphingomyelin to ceramide **(Lang et al., 2005c)**. Ceramide increases the sensitivity of erythrocytes to the excitatory effects of intracellular Ca^{2+} **(Lang et al., 2005c)**.

1.6. Calyculin A

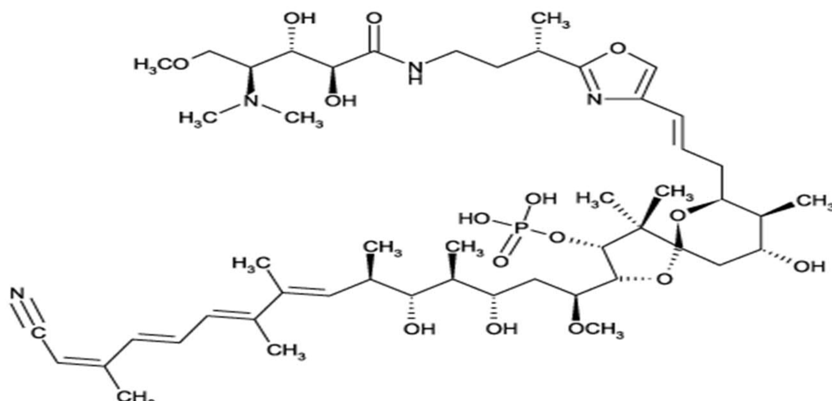

Fig. 1: Structure of the calyculin A backbone (Gotoh & Durante, 2006)

1.6.1. Calyculin A structure

Calyculin A is an exogenous xenobiotic and a natural marine sponge-derived metabolite that is present in mammalian cells, which was originally isolated from *Discodermia calyx*. It adopts a spiroketal skeleton shape and comprises amide, nitrile, oxazole, and phosphate. Calyculin A is a potent inhibitor of serine-threonine phosphatases, specifically protein phosphatase-1 (PP1) and protein phosphatase-2 (PP2) enzymes, which remove phosphate residues from protein scaffolds and play a major role in cell signaling pathways. In comparison to okadaic acid, which is a toxin produced by different specifies of dinoflagellates and an effective inhibitor of certain protein phosphatases, calyculin A has been demonstrated to be a more potent inhibitor. It represents a potential anticancer agent due to its ability to inhibit phosphatases enzymes **(Ishihara et al., 1989).**

1.6.2. Calyculin A biosynthesis

Entotheonella, a filamentous microorganism, is responsible for the biosynthesis of calyculins in *Discodermia calyx*. The production of calyculin requires the hydrolysis of a phosphate bond, which occurs through a yet unidentified phosphatase, the same enzyme class

inhibited by the natural product itself **(Habrant and Koskinen, 2010).** Calyculin A has been shown to have inhibitory effects on the endogenous phosphatase enzymes of the smooth muscle myosin B, resulting in smooth muscle fiber contraction. Calyculin A-induced contraction was led by a surge in the concentration of cytosolic Ca^{2+} **(Ishihara et al., 1989).** Furthermore, calyculin A induced apoptosis in human osteoblastic cells. In a previous study, calyculin A-induced apoptosis in osteoblastic MG63 cells as determined by the WST-8 assay **(Tanaka et al.2007)**. FasR mRNA, FasL, and PTEN expression were stimulated following calyculin A treatment. Furthermore, calyculin A enhanced the phosphorylation level of NF-kappaB **(Tanaka et al. 2007)**. Additionally, calyculin A plays a vital role in axonal transport impairment in neuroblastoma-2a (N2a) cells **(Liu et al., 2014)**. Selective inhibition of PP-2A and PP-1 with calyculin A has been demonstrated to not only cause hyperphosphorylation of cytoskeletal proteins, but also to impair the transport of the pEGFP-labeled NF-M subunit (EGFPNFM) in the axon-like processes of N2a cells and to result in the accumulation of neurofilaments in the cell bodies **(Yang et al., 2007).**

1.7. Camalexin

1.7.1. Camalexin structure

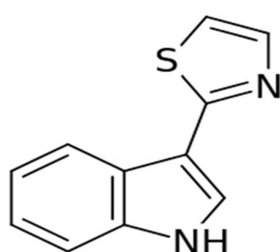

Fig. 2: Base structure of camalexin (Mezencev, Updegrove et al. 2011)

Camalexin is an exogenous xenobiotic and a major indole phytoalexin of *Arabidopsis thaliana* **(Pilatova et al., 2013)**. As shown in Figure 2, camalexin comprises of an indole ring that is derived from tryptophan, with an ethanamine moiety attached to the third

position of the indole ring. It accumulates in different cruciferous plants as a response to environmental stress **(Slusarenko and Schlaich, 2003)**.

Additionally, camalexin has been reported to exhibit potent antibacterial, antifungal, anti-proliferative, and cancer chemopreventive activities. Therefore, it plays a role in the defense against several pathogens. Its secondary metabolite serves as a phytoalexin, which fights against bacterial and fungal pathogens. In *Arabidopsis thaliana*, *Phytophthora capsici* shows resistance to camalexin and salicylic acid **(Wang et al., 2013)**. There is limited research on the cytotoxic effects of camalexin against eukaryotic cells and its application as a prospective drug for human diseases. Interestingly, camalexin has been recommended for the treatment of malignancies **(Mezencev et al., 2003) (Glawischnig, 2007)**. Camalexin limits the extension and spread of tumors, in part by encouraging suicidal death or cancer cell apoptosis. Several studies have demonstrated its cytotoxic effects on prostatic cancer cells, specifically with cells characterized by markedly increased activity and enhanced ROS levels **(Mezencev et al., 2011; Pilatova et al., 2013; Smith et al., 2014; Smith et al., 2013).**

1.7.2. Camalexin synthesis

During the biosynthesis of camalexin, a major group of enzymes that play a role in the biosynthetic pathway has been identified **(Schuhegger et al., 2007)**. The biosynthetic pathway begins with a tryptophan precursor. Two cytochrome p450 enzymes oxidize tryptophan, giving rise to indole-3-acetaldoxime, which is altered to indole-3-acetonitrile (Fig. 3) by cytochrome P450, CYP71A13 **(Glawischnig & Halkier, 2004)**. Dihydrocamalexic acid is formed after glutathione conjugation, followed by catalyzation with a subsequent unknown enzyme. The final step involves removing the carboxyl group by cytochrome P450 through CYP71B15, also termed phytoalexin deficient 3 (PAD3) **(Zhou & Glazebrook, 1999)**. After completing a series of processes and changes, the end product camalexin is formed **(Nafisi et al., 2007)**.

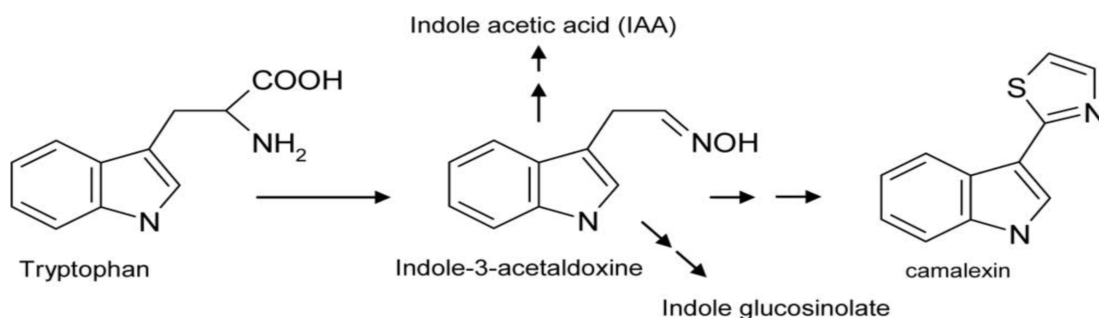

Fig. 3: Synthesis of camalexin from tryptophan (Iriti and Faoro 2009)

2. MATERIALS AND METHODS

The following materials and methods have been published previously **(Almasry, Jemaa et al. 2016) (Almasry, Jemaa et al. 2017)**.

2.1. Erythrocytes, solutions, and chemicals

All samples were provided by volunteers aged between 18 and 68 years. Samples were screened routinely for hepatitis A, B, and C, human immunodeficiency virus, and syphilis according to the guidelines of the blood bank of the University of Tübingen. This study was approved by the ethics committee of the University of Tübingen (ethics number 184/2003V) since volunteers provided informed consent for the use of blood samples for research purposes.

Blood samples were incubated *in vitro* at a hematocrit of 0.4% (pH 7.4) in Ringer's solution containing (125 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM KCl, 32 mM HEPES (PH 7,4), and 5 mM glucose) or in serum, as indicated at 37°C for 48 h. To investigate the role of calcium entry in phosphatidylserine exposure in erythrocytes treated with camalexin (MedChem Express, Princeton, USA) and calyculin A (MedChem Express, Princeton, USA), calcium-free Ringer solution was used. In an additional step, $CaCl_2$ was removed without the addition of a chelating agent (1 mM EGTA; Merck Millipore, Darm-

stadt, Germany). Following incubation of the erythrocytes in Ringer solution in the presence or absence of Ca^{2+}, samples were washed with Ringer solution containing 5 mM $CaCl_2$.

To assess the potential kinase activity, erythrocytes were exposed for 48 h to a combination of the drugs separately, as well as the protein kinase C inhibitor staurosporine (Sigma Aldrich, Hamburg, Germany), p38 kinase inhibitor SB 203580 (Tocris bioscience, Bristol, UK), or casein kinase inhibitor D4476 (Sigma Aldrich, Hamburg, Germany).

Caspase involvement was analysed by exposing erythrocytes for 48 h to a combination of treated erythrocytes and the pan-caspase inhibitors zVAD and zIETD-fmk (Tocris bioscience, Bristol, UK). Z-IETD-FMK belongs to the caspase family. It is an irreversible and cell-permeable inhibitor of caspase-8. Cell-permeable FMK peptide acts as an irreversible caspase inhibitor without cytotoxic effects. Therefore, they are useful in caspase activity studies. Furthermore, they play a key role in inflammation and mammalian apoptosis. Z-IETD-FMK can be used to inhibit caspase-8 activity and study events downstream of caspase-8 activation. Z-DEVD-FMK is an irreversible inhibitor of caspase-3/CPP32 that inhibits tumor cell apoptosis. It is relatively selective for caspase-3. In addition, it has been widely used in *in vitro* and *in vivo* models of acute injury to delineate roles for caspase 3 in neuronal cell death. Z-DEVD-FMK injections into the cerebral ventricles have been reported to improve function after LFP3. A marked compression in lesion volume was found after intraparenchymal injection over several days after combined CCI and hypoxia **(Clark et al., 2000)**.

2.2. FACS analysis of annexin V binding and forward scatter

After incubation, cells were washed in Ringer solution containing 5 mM $CaCl_2$. Erythrocytes were stained in annexin V-FITC (1:2000, Friesoythe, Germany) at 37°C for 20 min in the dark, followed by flow cytometric analysis on a FACS Calibur (Becton Dickinson, Heidelberg, Germany). Annexin V binding to erythrocytes was measured using an excitation wavelength of 488 nm and an emission wavelength of 530 nm. In addition, erythrocyte volume was determined via forward scatter light utilizing flow cytometry. Total events ranged from 10 000 to 15 000. For analysis and data acquisition,

the BD CellQuest Pro software (BD Biosciences) was used.

2.3. Intracellular Ca^{2+} measurement

Pre-incubated erythrocytes were washed with 50 µl of Ringer solution and loaded with fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo- 3/AM. Cells were incubated for 30 min at 37°C and washed subsequently two times in Ringer solution with 5 mM of $CaCl_2$. The fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer solution. Ca^{2+}-dependent fluorescence intensity was measured in the fluorescence channel (FL-1).

2.4. Measurement of hemolysis

Following incubation, the erythrocyte suspension was centrifuged for 3 min at 1600 rpm at 4°C, and the supernatant harvested. To determine hemolysis, the hemoglobin (Hb) concentration in the supernatant was measured photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

2.5. Ceramide abundance

Monoclonal antibody-based assays were used to determine ceramide abundance. 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. Subsequently, samples were washed twice with PBS-BSA. Cells were then stained for 30 min with a 1:50 dilution of polyclonal fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) in PBS-BSA. Unbound secondary antibody was removed by frequent washing with PBS-BSA. The samples were analyzed by flow cytometric analysis using the fluorescence channel (FL-1).

2.6. ROS measurement

Oxidative stress was determined using the fluorogenic dye 2',7' -dichlorofluorescein diacetate (DCFDA). After incubation, a 50 µl suspension of the erythrocytes was washed in Ringer solution, followed by staining with DCFDA (Sigma, Schnelldorf, Germany) in Ringer solution containing a final DCFDA concentration of 10 µM. Erythrocytes were incubated for 30 min at 37°C in the dark. Cells were subsequently washed three times in Ringer solution. The erythrocytes loaded with DCFDA were suspended in 200 µl Ringer solution. ROS-dependent fluorescence intensity was measured using the fluorescence channel (FL-1) on a FACS Calibur (BD).

2.7. Statistics

Statistical analysis was performed using ANOVA with Tukey's tests. Data are expressed as arithmetic means ± SEM. N denotes the number of different erythrocyte specimens studied. Identical erythrocyte specimens were used in both control and experimental conditions.

3. Results

We applied two different xenobiotics, calyculin A and camalexin, on human erythrocytes to investigate their effect on eryptosis, major contributory apoptotic pathways, and signaling pathways involved in their mediation. Accordingly, FACS was applied to measure fluorescent and light scattering cellular properties. Annexin V binding, forward scatter, and Fluo-3 fluorescence was used as a proxy for cell surface phosphatidylserine exposure, cell volume, and intracellular Ca^{2+}concentration, respectively. The results of these measurements for the two drugs are detailed in the following sections.

3.1. Effect of calyculin A on eryptosis

Calyculin A exposure causes erythrocyte shrinkage

One of the major morphological indicators of eryptosis is erythrocyte volume shrinkage. To determine changes in the erythrocyte volume after treatment with calyculin A, cells were assessed by flow cytometry. Samples were incubated for 48 h in Ringer solution in the presence or absence of 2.5–10 nM of calyculin A. As indicated in Fig. 4, calyculin A significantly decreased the forward scatter of all analyzed concentrations, with a maximum decrease observed at 10 nM.

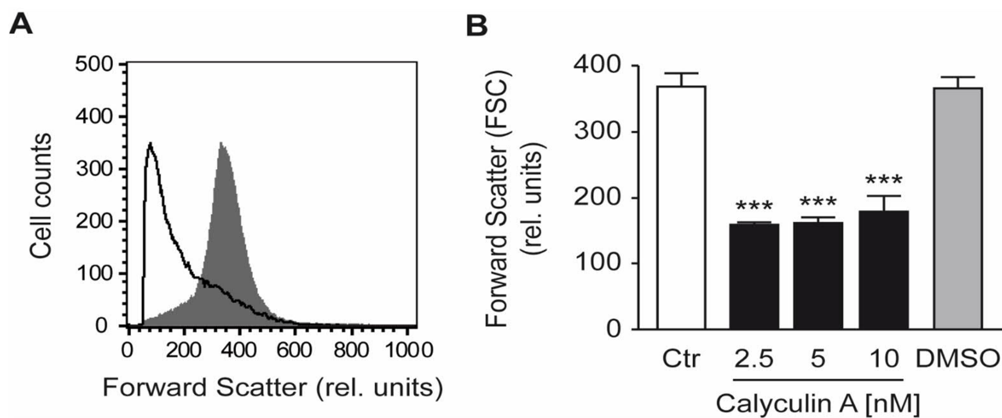

Fig. 4: Effect of calyculin A on erythrocyte forward scatter. (A) Histogram of the forward scatter of erythrocytes from healthy blood donors after incubation with Ringer solution (grey) or in the presence of 2.5 nM to 10 nM calyculin A (indicated as a black line). **(B)** The effect of variable concentrations of calyculin A on the forward scatter. The arithmetic mean ± SEM (n = 10) of the normalized forward scatter of erythrocytes incubated for 48 h with Ringer solution (white bar) or various concentrations of calyculin A (black bars) are indicated. *** A p value less than 0.001 indicates a significant change compared to control values (ANOVA). DMSO (grey) was used as a comparative control.

Calyculin A increases surface phosphatidylserine exposure

Phospholipid scrambling on the plasma membrane with subsequent phosphatidylserine translocation to the cell surface is another hallmark of eryptosis. FACS was utilized to determine the phosphatidylserine exposing erythrocytes via fluorescent annexin V. Accordingly, erythrocyte exposure to calyculin A for 48 h enhanced the percentage of annexin-V-binding erythrocytes (Fig. 5).

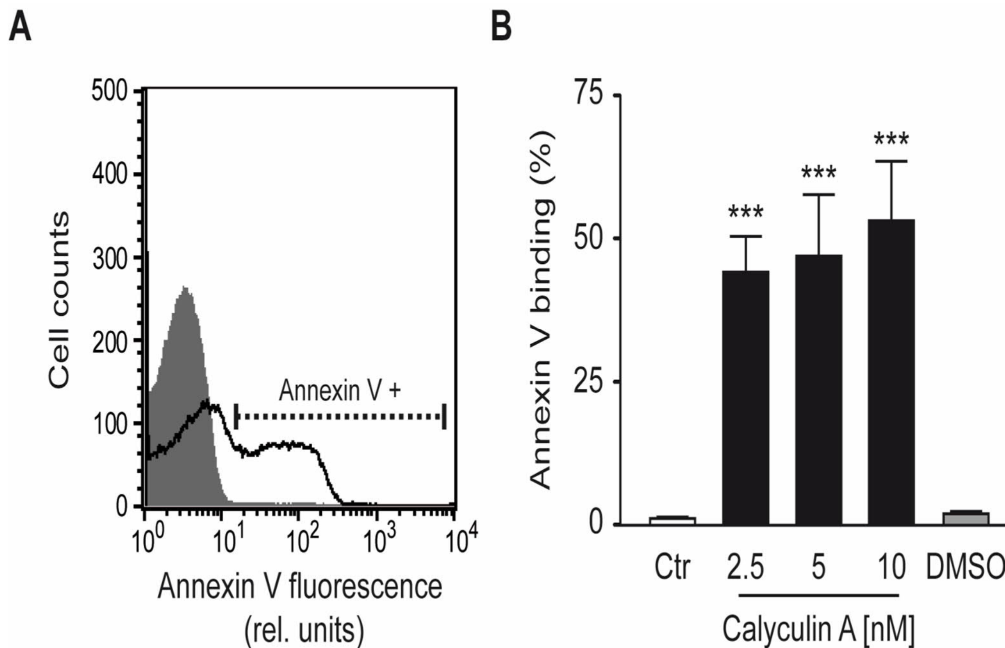

Fig. 5: Effect of calyculin A on phosphatidylserine exposure on the outer surface of erythrocytes and hemolysis. (A) Histogram of annexin V binding of erythrocytes from healthy blood donors. Cells were incubated for 48 h with Ringer solution (grey) or in the presence of 2.5–10 nM calyculin A (indicated as a black line). **(B)** Effect of calyculin A on phosphatidylserine exposure on the outer surface and hemolysis. The arithmetic mean ± SEM (n = 10) of the percentage of erythrocytes bound to annexin V after incubation for 48 h with Ringer solution (white bar) or calyculin A (black bars). The arithmetic mean ± SEM (n = 4) was used to express the percentage of hemolysis (grey bars). *** A p value less than 0.001 indicates a marked surge compared to control values in Ringer solution (ANOVA). DMSO (grey) was used as a control.

Calyculin A increases calcium influx into eryptotic cells

Erythrocytes were incubated with fluo-3-AM and Ringer solution in the presence and absence of calyculin A (2.5–10 nM), followed by FACS analysis. The intracellular Ca^{2+} concentration was assessed via fluo-3 fluorescence to unveil mechanisms responsible for triggering erythrocyte volume compression and membrane scrambling following calyculin A exposure.

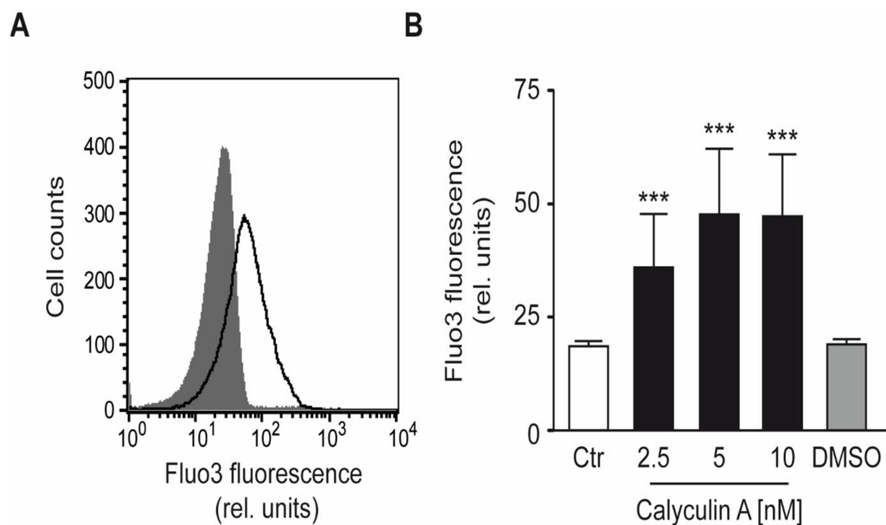

Fig. 6: Effect of calyculin A on erythrocyte cytosolic Ca^{2+} concentration.: (A) Histogram of fluo-3 fluorescence of erythrocytes from healthy blood donors after incubation with Ringer solution (grey) in the presence of 2.5 nM to 10 nM calyculin A (represented as a black line) for 48 h. **(B)** The effect of variable concentrations of calyculin A on fluo-3 fluorescence. The arithmetic means ± SEM (n = 10) of the normalized fluo-3 fluorescence of erythrocytes incubated for 48 h with Ringer solution (white bar) or various concentrations of calyculin A (black bars) are indicated. ***A p value less than 0.001 indicates a significant surge of the test sample compared to the control solution. Statistical analysis was performed using ANOVA. DMSO (grey) was used as a comparative control.

Incubation with calyculin A was observed to significantly increase the fluorescence intensity of the erythrocytes, indicating a surge in the cytosolic Ca^{2+} concentration at each concentration tested (Fig. 6).

Calyculin A-induced cellular calcium influx is important for the eryptotic mechanism

To explore the significance of calyculin A-induced Ca^{2+} entry, 10 nM of calyculin A was analyzed in the presence and absence of extracellular Ca^{2+}. As shown in Fig. 7, the effect of calyculin A on phosphatidylserine exposure resulted in significant attenuation of phosphatidylserine exposure in the absence of extracellular Ca^{2+}.

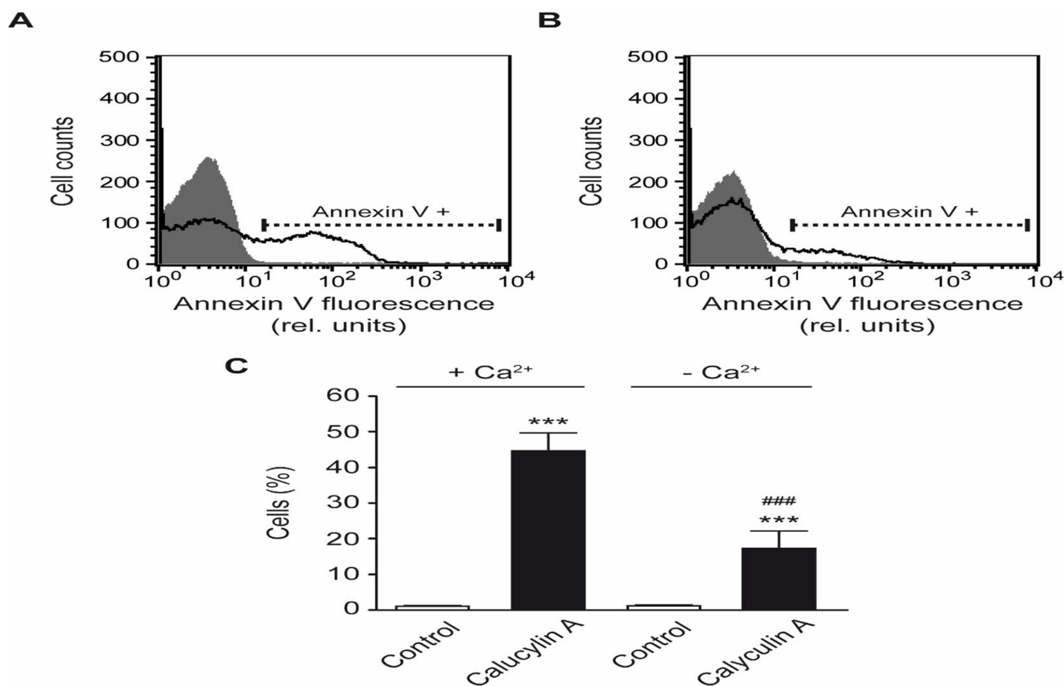

Fig. 7: Effect of Ca^{2+} withdrawal on erythrocytes bound to calyculin A in terms of annexin-V binding. The percentages of erythrocytes bound to annexin V are expressed as an arithmetic mean ± SEM (n = 10) 48 h following exposure to Ringer solution (white bars) and 10 nM calyculin A (black bars) in the presence (left bars) or absence (right bars) of Ca^{2+} in extracellular fluid. *** A p value less than 0.001 indicates a significant difference to control values. ### A p value less than 0.001 indicates a marked difference to other samples in the presence of Ca^2. Statistical analysis was performed using ANOVA.

The amount of phosphatidylserine exposed at the outer surface of calyculin A treated erythrocytes was markedly higher in the presence of extracellular Ca^{2+}. However, an increase was observed in erythrocytes without Ca^{2+} compared to the control. Therefore, eryptosis was triggered principally, yet not exclusively as a result of calcium entry. This indicated other mechanisms contributing to calyculin A-induced eryptosis.

Caspases and kinases involved in the molecular mechanism of calyculin A-induced eryptosis

To reveal the sensitivity of calyculin A-induced phosphatidylserine exposure to kinase and caspase activity, the effect of calyculin A on annexin-V-binding was examined using the protein kinase C inhibitor staurosporine (1 µM), p38 kinase inhibitor SB203580 (2 µM), casein kinase inhibitor D4476 (10 µM), or caspase inhibitor zVAD (10 µM). As shown in Fig. 8, the effect of calyculin A (10 nM) on phosphatidylserine exposure was significantly decreased in the presence of SB203580, staurosporine, D4476 and zVAD.

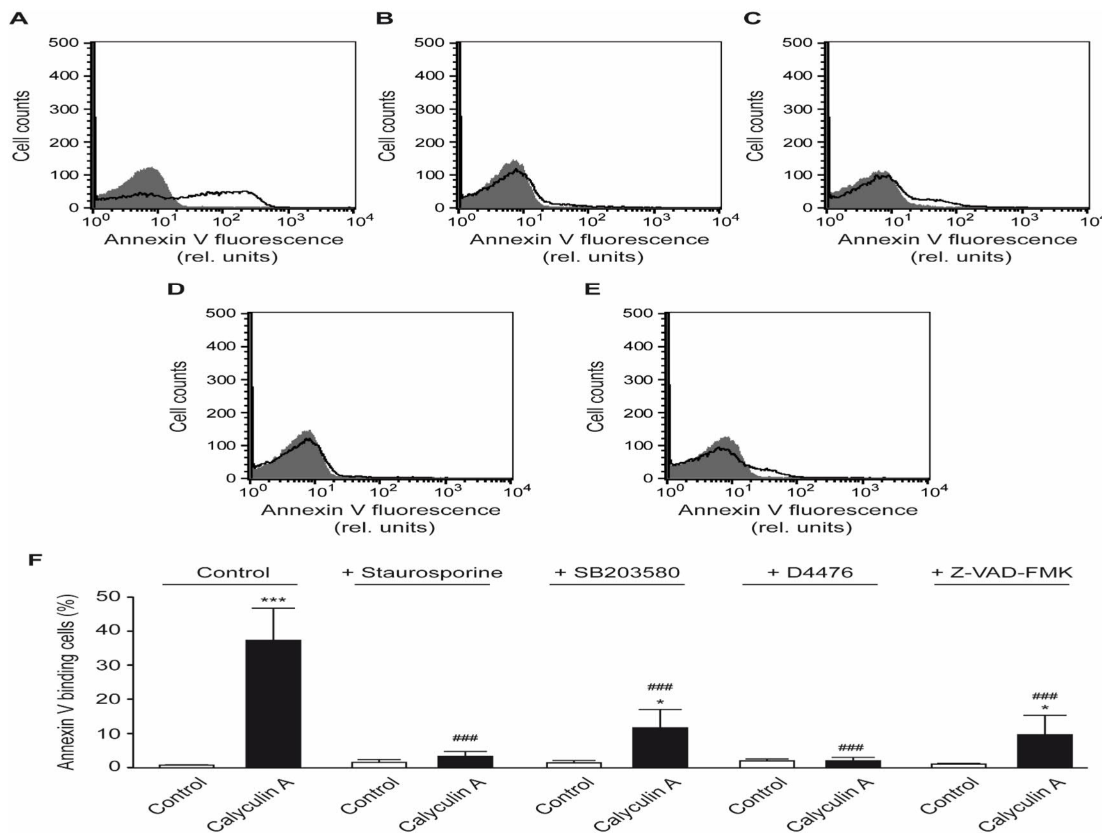

Fig. 8: Effect of SB203580, staurosporine, and D4476 on calyculin A-induced annexin V binding. (A)–(E) Histograms of annexin V binding of erythrocytes after incubation for 48 h with Ringer solution in the absence (grey) or presence (black lines) of calyculin A (10 nM) with (A) or without 1 µM staurosporine (B), 2 µM SB203580 (C), 10 µM D4476 (D), or 10 µM zVAD (E). (F). The arithmetic mean ± SEM (n = 10) of erythrocytes bound to annexin V after incubation for 48 h with Ringer solution in the absence (white bars) or presence (black bars) of calyculin A (10 nM) without (left bars, control) or with (right bars) staurosporine (+staurosporine), SB203580 (+SB203580), D4476 (+D4476), or zVAD (+Z-VAD-FMK). *(p < 0.05) and ***(p < 0.001) indicates a significant difference compared to the absence of calyculin A. ### (p < 0.001) indicates a significant difference compared to the absence of inhibitors. Statistical analysis was performed using ANOVA.

3.2. Effect of camalexin on eryptosis

Camalexin exposure causes erythrocyte shrinkage

As depicted in Figures 9A and 9B, a decrease in the forward scatter was observed following treatment with camalexin. All concentrations employed resulted in a significant decrease in the forward scatter. Hence, erythrocyte volume shrinkage occurred as a result of camalexin treatment.

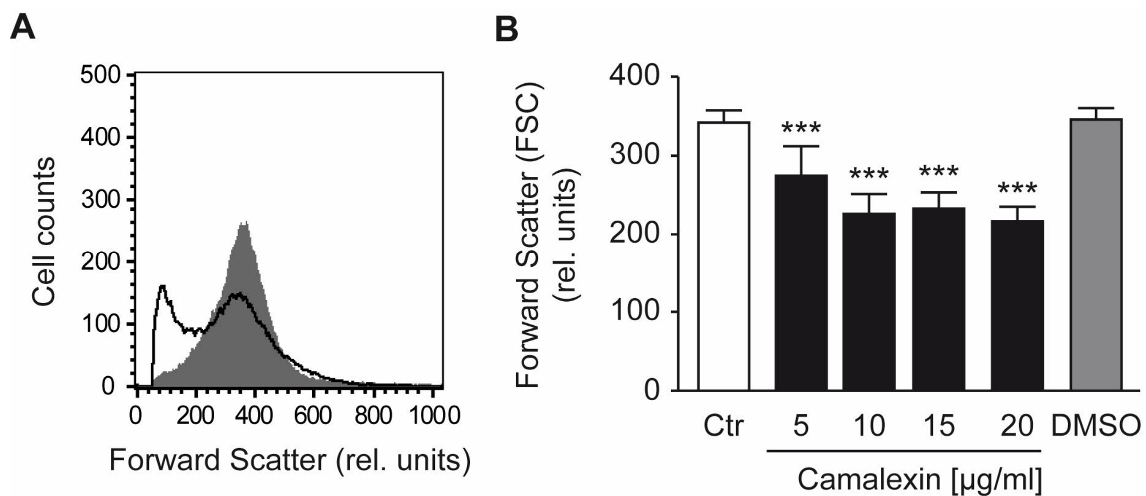

Fig. 9: Effect of camalexin on erythrocyte volume. (A) Histogram of FACS analysis of erythrocytes following incubation for 48 h with Ringer solution (grey) and 5–20 µg/ml camalexin (black line) **(B)** FACS analysis of the effect of camalexin. The arithmetic mean ± SEM (n = 10) of the forward scatter of erythrocytes incubated for 48 h with Ringer solution (white bar) and camalexin (black bars). ***($p < 0.001$) indicates a significant difference compared to control values (Ringer solution). DMSO (grey bar) was used as a control. Statistical analysis was performed using ANOVA.

Camalexin increases surface phosphatidylserine exposure

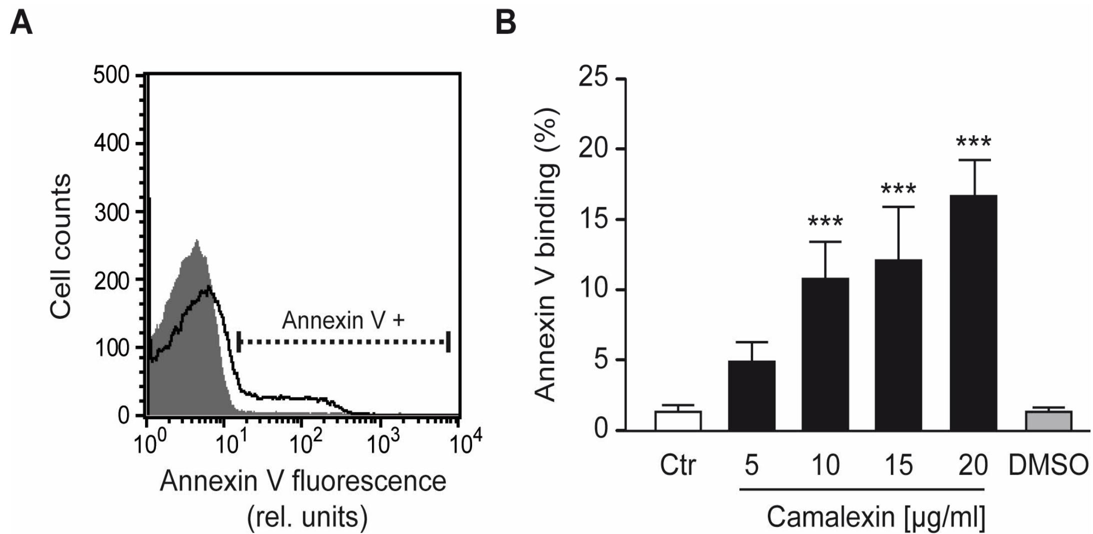

Fig. 10: Effect of camalexin on phosphatidylserine exposure and erythrocyte membrane integrity. (A) Histogram of annexin V fluorescence of erythrocytes incubated for 48 h with Ringer solution (grey) and following exposure to 20 µg/ml camalexin (black line). (Almasry, Jemaa et al. 2017) **(B)** Effect of camalexin on phosphatidylserine exposure. The arithmetic mean ± SEM (n = 12) of the percentage of annexin V bound erythrocytes incubated for 48 h with Ringer solution (white bar) and camalexin (black bars). DMSO (grey) was used as a control. *** ($p < 0.001$) indicates a significant positive change compared to the absence of the camalexin. Statistical analysis was performed using ANOVA. DMSO (grey) was used as a control.

To measure the role of camalexin on phosphatidylserine exposure and erythrocyte membrane integrity, the fluorescent annexin V was used to measure the percentage of phosphatidylserine exposure in untreated and camalexin treated erythrocytes. Accordingly, a camalexin concentration of ≥10 µg/ml had a significant effect on the binding of annexin V to erythrocytes (Figures 10A and 10B). The maximum effect was observed using the highest camalexin concentration used, 20 µg/ml. To examine whether camalexin induces hemolysis, the concentration of hemoglobin in the cell supernatant was determined. Exposure to 20 µg/ml camalexin did not increase the percentage of hemolytic cells (5.1 ± 0.8%, n = 4) compared to its absence (3.5 ± 0.8%, n = 4). Therefore, whereas camalexin significantly increased eryptosis, it did not significantly increase hemolysis.

Camalexin increases calcium influx into eryptotic cells

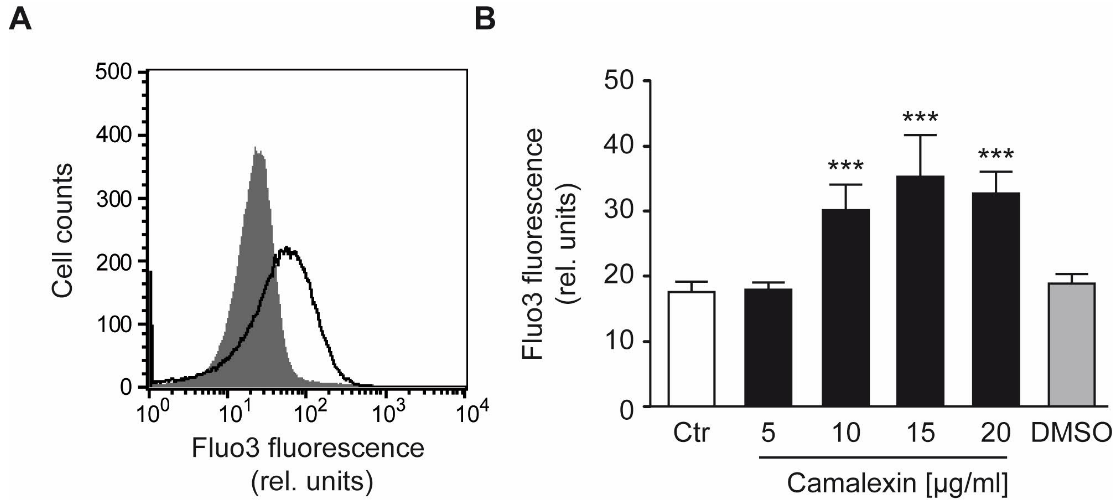

Fig. 11: Effect of camalexin on the erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo-3 fluorescence reflecting cytosolic Ca2+ concentration in erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 20 µg/ml Camalexin. B. The arithmetic mean ± SEM (n = 10) of the forward scatter of erythrocytes incubated for 48 h with Ringer solution (white bar) and camalexin (black bars). ***($p < 0.001$) indicates a significant difference compared to control values (Ringer solution). DMSO (grey) was used as a control. Statistical analysis was performed using ANOVA.

Fluo-3 fluorescence was used to investigate the role of camalexin on cytosolic Ca^{2+} concentration. As depicted in Fig. 11, there was a significant difference observed for erythrocytes incubated with camalexin (10–20 µg/ml) compared to the control containing only Ringer solution. The fluo-3 fluorescence was found to be directly proportional to the presence of camalexin as well as its concentration. Increasing the camalexin concentration resulted in an elevated fluo-3 fluorescence. A marked statistical significance was reached at 10 µg/ml camalexin.

Camalexin-induced cellular calcium influx is important for the eryptotic mechanism

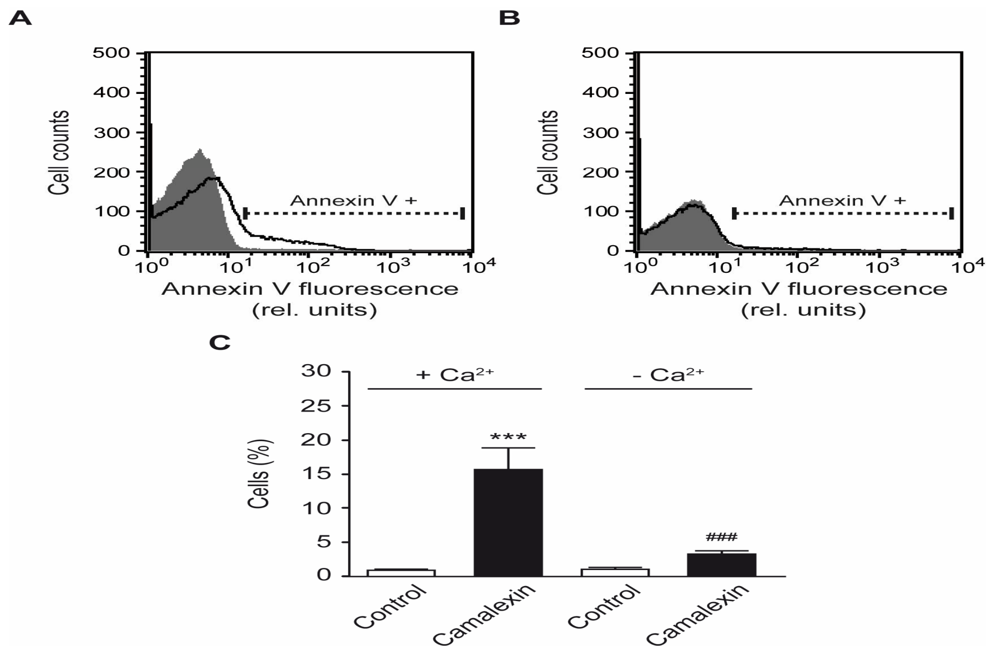

Fig. 12: Role of Ca^{2+} in camalexin-induced phosphatidylserine exposure. **(A)** and **(B)** Histograms demonstrating annexin V binding of erythrocytes after incubation for 48 h with Ringer solution in the presence (grey) or absence of camalexin (black line) with **(A)** or without Ca^{2+} **(B)**. **(C)** The arithmetic mean ± SEM (n = 10) after 48 h incubation with Ringer solution (white bars) or 20 µM camalexin (black bars) in the presence (left) or absence (right) of Ca^{2+}. *** ($p < 0.001$) indicates a significant difference compared to control values (Ringer solution). ### ($p < 0.001$) indicates a significant difference compared to other values in the presence of Ca^{2+}. Statistical analysis was performed using ANOVA.

To explore the role camalexin-induced cellular calcium influx in camalexin-induced phosphatidylserine exposure, erythrocytes were incubated for 48 h in the absence or presence of 20 µg/ml camalexin and in the presence or absence of extracellular Ca^{2+}. As depicted in Fig. 12, the absence of extracellular Ca^{2+} led to a significant decrease in annexin V-binding to erythrocytes. This indicates the involvement of calcium entry in camalexin-induced phosphatidylserine exposure. Thus, entry of extracellular Ca^{2+} contributes, at least partially, to camalexin-induced redistribution of phosphatidylserine in the cell membrane.

Caspases and kinases involved in the molecular mechanism of camalexin-induced eryptosis

To further investigate whether camalexin-induced eryptosis involved kinases, the effect of camalexin on phosphatidylserine asymmetry was tested. Annexin V-binding was measured after the exposure of (20 µg/ml) camalexin-treated erythrocytes for 48 h to kinase inhibitors; protein kinase C inhibitors staurosporine (1 µM), and chelerythrine (10 µM), p38 kinase inhibitor SB 203580 (2 µM) or casein kinase inhibitor D4476 (10 µM).

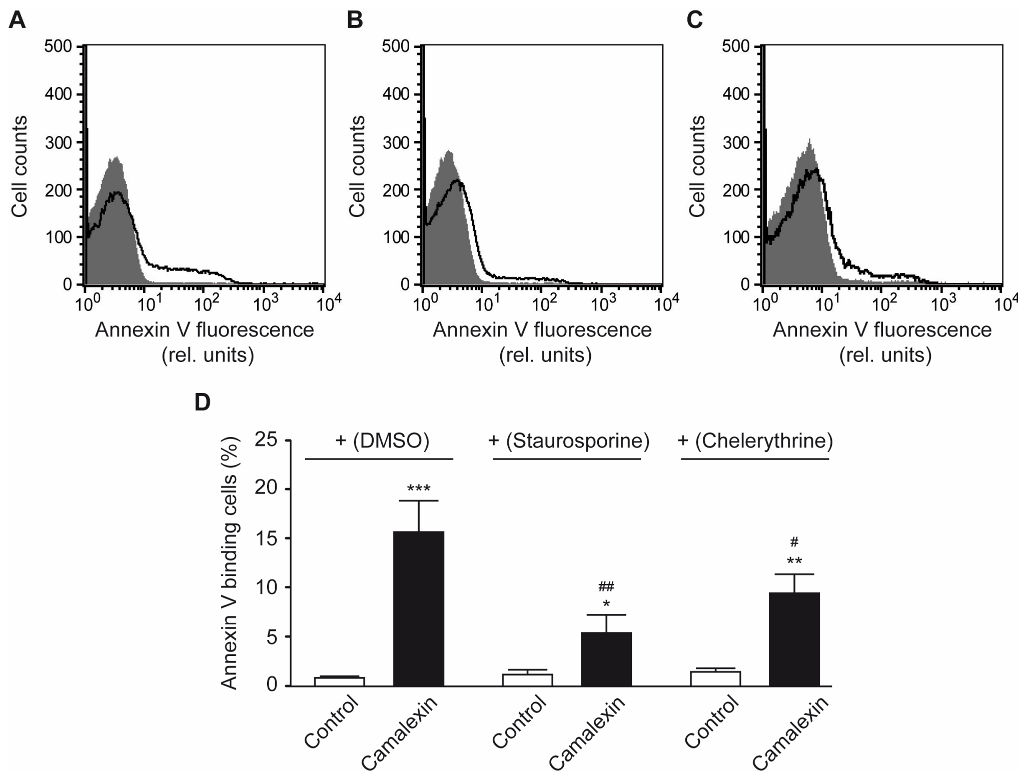

Fig. 13: Effect of staurosporine and chelerythrine on camalexin -induced phosphatidylserine exposure. (A) and **(B)** Histograms demonstrating annexin V binding of erythrocytes following incubation for 48 h with Ringer solution without (grey) and with (black lines) camalexin (20 µg/ml) in the absence **(A)** or presence of **(B)** 1 µM staurosporine or **(C)** 10 µM chelerythrine. The arithmetic mean ± SEM (n = 8) of erythrocytes bound to annexin V after incubation for 48 h with Ringer solution without (white bars) or with (black bars) camalexin (20 µg/ml) in the absence (left, + DMSO) or presence of 1 µM staurosporine (middle, + Staurosporine), or 10 µM chelerythrine (right, + Chelerythrine). *(p < 0.05) and *** (p < 0.001) indicate a significant difference compared to samples without camalexin, ## (p < 0.01) indicates a significant difference compared to samples without the corresponding kinase inhibitors. Statistical analysis was performed using ANOVA.

Fig. 13 depicts the effects of camalexin on annexin-V-binding as well as following the addition of chelerythrine (10 µM) and staurosporine (500 nM). Chelerythrine and staurosporine resulted in a significant decrease in annexin-V-binding. However, the ratio of annexin-V-binding erythrocytes after the exposure to 20 µg/ml camalexin was almost similar in the absence of inhibitors (15.7 ± 3.0 a.u., n = 10) and in the presence of inhibitors; either SB203580 (11.3 ± 6.8 a.u., n = 10, data not shown) or D4476 (12.4 ± 4.5 a.u., n = 10, data not shown). Therefore, the camalexin-induced phosphatidylserine asymmetry seemingly required the activation of staurosporine and chelerythrine sensitive kinases but not SB203580 sensitive or D4476 sensitive kinases.

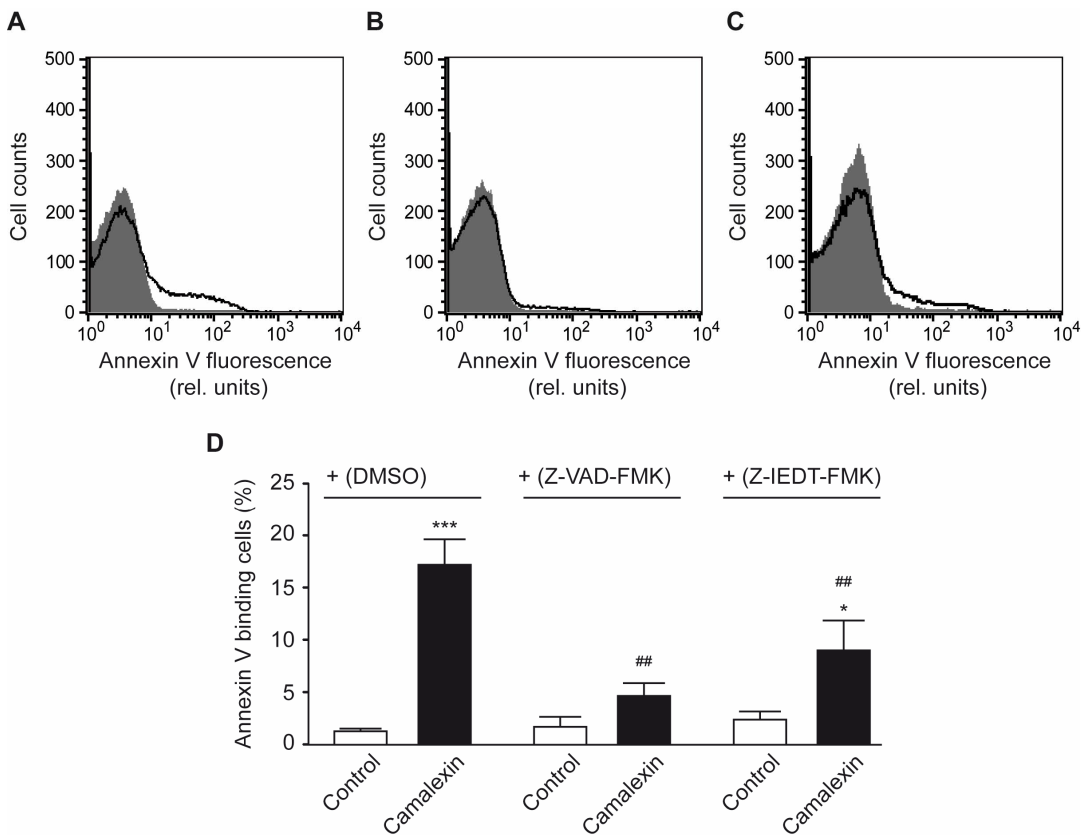

Fig. 14: Sensitivity of camalexin-induced phosphatidylserine exposure after zVAD- and zIETD-FMK treatment. (A) and **(B)** Histograms of erythrocytes bound to annexin V after incubation for 48 h with Ringer solution; without (grey areas) or with (black lines) camalexin (20 µg/ml) in the absence (A) or presence of 10 µM of the pan-caspase inhibitor zVAD-FMK (B) or 50 µM of the caspase 8 inhibitor zIETD-FMK **(C)**. The arithmetic mean ± SEM (n = 10) of erythrocytes bound to annexin V after incubation for 48 h with Ringer solution without (white bars) or with (black bars) camalexin (20 µg/ml) in the absence (left, DMSO) or presence of 10 µM zVAD-FMK (middle, + zVAD), or 50 µM zIETD-FMK (right bars, + zIETD-FMK). *** ($p < 0.001$) indicates a significant difference compared to samples without camalexin. ## ($p < 0.01$) indicates a significant difference compared to samples without the respective caspase inhibitor. Statistical analysis was performed using ANOVA.

Further experiments were performed as shown in Fig. 14 using various protein kinase inhibitors including pancaspase inhibitor zVAD (10 µM), caspase-8 inhibitor zIETD-fmk (50 µM), and caspase-3 inhibitor zDEVD (50 µM). Camalexin-induced cell membrane asymmetry was further significantly decreased in the presence of pan-caspase inhibitor zVAD and caspase 8 inhibitor zIETD-fmk. Hence, camalexin-induced eryptosis appeared to require the activation of zVAD and zIETD-fmk sensitive caspases. However, in the absence of inhibitors (16.9 ± 2.9 a.u., n = 8) and in the presence of zDEVD-fmk (19.1 ± 1.9 a.u., n = 8, data not shown), the percentage of annexin-V-binding erythrocytes following exposure to 20 µg/ml camalexin was similar.

4. DISCUSSION

The presence of certain foreign substances in the blood has the potential to damage erythrocytes and trigger eryptosis **(Lang and Wieder, 2006) (Akel and Lang, 2006)**. Eryptosis leads to the rapid clearance of phosphatidylserine exposing erythrocytes from the circulating blood. Consequently, anemia may occur once erythrocyte loss outweighs the process of erythropoiesis **(Lang & Lang, 2015b)**.

Furthermore, the inhibition of eryptosis may prove beneficial for the treatment of certain clinical conditions where overstimulation of eryptosis is evident. Examples include sickle cell anemia or malaria, whereby overstimulation may exacerbate anemia in affected patients **(Föller and Lang, 2008) (Föller and Lang, 2009) (Hermle and Lang, 2006)**. Hence, investigating eryptotic inhibitors may be significant to adjust erythrocyte homeostasis.

4.1. Calyculin A involvement in the suicidal death of erythrocytes

The present study aimed to determine the effect of calyculin A on eryptosis. Calyculin A was found to be an eryptotic inducer **(Almasry, Jemaa et al. 2016)**. A concentration range of 5–10 nM was determined to be a sufficient apoptotic trigger, which is comparable to concentrations (2.5–10 nM) observed in a previous study **(Tanaka et al. 2007)**. The eryptotic effect was strongest at the maximum concentration of 10 nM calyculin A. Moreover, we ascertained that the increase of cytosolic Ca^{2+} activity in erythrocytes following calyculin A treatment was an important mechanism of calyculin A–stimulated eryptosis. This finding is consistent with the previously characterized molecular mechanism of eryptosis **(Montague et al., 1999)**, which delineated the role of Ca^{2+} influx in cell volume compression. This occurs through the activation of Ca^{2+}-specific potassium channels, resulting in cell membrane hyperpolarization, Cl^- ion exit, and, therefore, the cellular efflux of KCl with water. In corroboration, we found that the absence of extracellular Ca^{2+} significantly attenuated phosphatidylserine asymmetry (a hallmark of eryptosis) and erythrocyte cell membrane shedding, highlighting the need for Ca^{2+} influx for calyculin A–induced eryptosis. Nevertheless, removal of extracellular Ca^{2+} did not completely abolish calyculin A–induced eryptosis. This observation suggests that

different triggering mechanisms, apart from an augmented cytosolic Ca^{2+} activity, might additionally be at play in calyculin A-induced eryptosis.

The results of the present study demonstrate that the effect of calyculin A on eryptosis was markedly decreased by the protein kinase C inhibitor staurosporine and the casein kinase inhibitor D4476. In addition, there was a slight but significant reduction on the calyculin A-induced phosphatidylserine exposure by the p38 kinase inhibitor SB203580, and the caspase inhibitor zVAD **(Almasry, Jemaa et al. 2016)**. These observations suggest that these enzymes may act downstream of calyculin A sensing by erythrocytes, and identifies them to be involved in the calyculin A-induced eryptotic pathway.

4.2. Modulation of erythrocyte survival by camalexin

The present study demonstrated camalexin to be an inducer of eryptosis. However, camalexin did not significantly modify hemolysis at the analyzed concentrations **(Almasry, Jemaa et al. 2017).**

The effect of camalexin on phospholipid scrambling of the erythrocyte cell membrane depended primarily on Ca^{2+}influx from the extracellular space. The removal of extracellular Ca^{2} significantly attenuated the effect of camalexin on phosphatidylserine redistribution**.** The entry of Ca^{2+} from the extracellular space into the erythrocytes could further account for the camalexin-induced cell shrinkage, which could result from an increase of intracellular Ca^{2+}, with subsequent activation of Ca^{2+} specific K^{+} channels, K^{+} efflux, cell membrane hyperpolarization, Cl^{-} exit, and, therefore, cellular loss of KCl^{-} in addition to water.

Specific protein kinase inhibitors affected the potency of camalexin on eryptosis. While eryptosis was significantly blunted in the presence of the protein kinase C inhibitor staurosporine, this effect was not observed for the p38 kinase inhibitor SB203580 or the casein kinase inhibitor D4476. This means that the latter two kinases, but not the former, are components of the molecular pathway involved in camalexin-induced eryptosis. Camalexin-induced cell membrane phospholipid scrambling was further considerably attenuated in the presence of the pan-caspase inhibitor zVAD. Therefore, caspase activation likely represents one of the mechanisms involved in camalexin-induced

eryptosis. This is consistent with the well-known involvement of caspase activation cascades in apoptosis **(Berg et al., 2001)**. In conclusion, the present study showed that increased cytosolic Ca^{2+} levels, as well as staurosporine-sensitive kinase, and caspase activity are notable triggers of camalexin-induced eryptosis **(Almasry, Jemaa et al. 2017)**. On the other hand, the roles played by ceramide abundance and oxidative stress in camalexin-induced eryptosis were not found to be significant **(Almasry, Jemaa et al. 2017)**.

In summary, both calyculin A and camalexin were identified as novel eryptotic inducers. Both shared similar effects and increased the classical hallmarks of eryptosis such as elevated intracellular Ca^{2+}, cell volume shrinkage, and phosphatidylserine exposure. However, calyculin A and camalexin differed in the identity of the enzymes involved in transducing the eryptotic signal. This enabled us to characterize an important possible toxicological mechanism, and provides evidence that these xenobiotics should be avoided in clinical pathologies already characterized by excessive eryptosis. Excessive eryptosis can result in the coherence of phosphatidylserine-exposing erythrocytes to endothelial CXCL16/SR PSO **(Borst et al., 2012)**. Therefore, the resulting interference with blood flow may be compromised by the adherence of erythrocytes to the vascular wall. The impact is potentially compounded by the stimulating effect of phosphatidylserine exposure on blood clotting, which may lead to thrombosis **(Andrews & Low, 1999) (Closse, Dachary-Prigent, & Boisseau, 1999)**. To the extent that the effective concentrations are reached in the plasma of camalexin-treated patients and calyculin A-treated patients, eryptosis could increase the risk of thromboembolism and anemia.

5. SUMMARY

Eryptosis, the suicidal death of erythrocytes (red blood cells), is known to be affected by xenobiotics. A characteristic hallmark of eryptosis is increased intracellular calcium (Ca^{2+}), which opens Ca^{2+}-dependent K^+ channels resulting in cellular loss of KCl, and the associated osmotic efflux of water, resulting in cell shrinkage. Furthermore, molecular eryptotic changes culminate in cell membrane scrambling, exposing phosphatidylserine on the outer membrane leaflet. Here, we investigated the effect of two drugs, calyculin A and camalexin, on eryptosis. Fluorescence activated cell sorting (FACS) and light scattering were applied to measure changes in fluorescent properties and the size of erythrocytes after treatment. Fluo3 fluorescence and annexin V-binding were used as a proxy for the intracellular Ca^{2+} concentration and phosphatidylserine exposure, respectively.

Insights on the effects of calyculin A on eryptosis were investigated. Calyculin A was found to increase cytosolic Ca^{2+} activity and phosphatidylserine surface presentation, implicating it as an eryptotic inducer. The eryptotic induction effect of calyculin A was abolished in the absence of extracellular Ca^{2+} ions, highlighting its dependence on Ca^{2+} entry. On the other hand, additional experiments demonstrated that ceramide abundance, another property frequently occurring in eryptotic cells, was not significantly modified. Finally, staurosporine, SB203580, D4476, and zVAD, inhibitors of kinases and caspases, markedly blunted the effect of calyculin A on annexin V-binding. This indicated the involvement of the respective enzymes in the specific molecular pathways that mediate calyculin A-induced eryptosis.

Similarly, camalexin was found to significantly increase cytosolic Ca^{2+} activity due to the activation of Ca^{2+} permeable cation channels, and cause subsequent cell shrinkage as well as increased membrane phosphatidylserine redistribution. However, the effect of camalexin was not abolished entirely upon the removal of extracellular Ca^{2+} ions, indicating the involvement of another mechanism. Camalexin-induced phosphatidylserine surface exposure has been previously shown to involve staurosporine- and chelerythrine-sensitive kinases, such as protein kinase C. Interestingly, cell membrane scrambling was abolished in the presence of staurosporine and chelerythrine but not the p38 kinase inhibitor SB203580 and casein kinase inhibitor D4476, suggesting the involvement of protein

kinase C in the molecular eryptotic pathway induced by camalexin. Additionally, general caspase inhibitors, such as Z-IETD-FMK, a caspase-8 inhibitor, and Z-DEVD-FMK, a caspase-3 inhibitor, were found to attenuate camalexin-stimulated eryptosis.

In conclusion, the two studies presented in this work reveal that calyculin A and camalexin are eryptotic inducers as demonstrated by their ability to incite cell volume shrinkage and phospholipid scrambling on the plasma membrane. Moreover, this effect is dependent on Ca^{2+} influx in the case of calyculin A, and, at least, partially dependent on Ca^{2+} influx for camalexin. While resulting in the same phenotypic outcomes, calyculin A- and camalexin-induced eryptosis differed in the exact enzymes and pathways that transduce the eryptotic signal.

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7. GERMAN SUMMARY

Der Schwerpunkt dieser Studie liegt auf der Eryptose, dem suizidalen Tod von Erythrozyten nach der Behandlung mit bestimmten Arzneimitteln. Es wurden die Auswirkungen von Calyculin A und Camalexin auf die Eryptose untersucht. Um die Erythrozyten-Größe (durch Forward Scatter Licht,) die zytosolische Ca^{2+} -Konzentration (durch Fluo3-Fluoreszenz) und die Phosphatidylserintranslokation (durch Annexin V-Bindung) zu untersuchen, wurde FACS Analyse verwendet.

Nach Verabreichung von Calyculin A, eine deutliche Erhöhung der zytosolischen Ca^{2+} -Konzentration festgestellt. In dieser Studie ist zytosolisches Ca^{2+}vermutlich ein Hauptauslöser der Eryptose. Bemerkenswerterweise wird dieser eryptotische Effekt in Abwesenheit von Ca^{2+}aufgehoben. Darüber hinaus haben protein kinase C inhibitor staurosporine (1 µM), p38 kinase inhibitor SB203580 (2 µM), casein kinase inhibitor D4476 (10 µM), und caspase inhibitor zVAD (10 µM) die Wirkung von Calyculin A auf die Annexin V-Bindung signifikant abgeschwächt.

Die vorliegende Arbeit untersucht auch die Rolle von Camalexin bei der Stimulierung der Apoptose und zeigt die zugrundeliegenden Mechanismen. Camalexin erhöht die zytosolische Ca^{2+}-Aktivität aufgrund der Aktivierung von Ca^{2+}-permeablen Kationenkanälen. Dies führt zu einer Aktivierung von Ca^{2+}-spezifischen K+ -Kanälen und wird von verschiedenen zellulären Ereignissen, wie dem Verlust von KCl, die den Ausfluss von osmotisch gebundenem Wasser und schließlich das Schrumpfen der Zellen verursacht, begleitet. Die Wirkung von Camalexin wird durch die Entfernung extrazellulärer Ca^{2+}-Ionen abgeschwächt aber nicht aufgehoben. Camalexin ist daher teilweise wirksam, indem es den Ca^{2+}-Einstrom stimuliert. Abgesehen von der Hauptrolle von Ca^{2+} bei der Auswirkung von Camalexin auf die Eryptose, wirken mehrere Kinasen mit.Kinase.Hemmer Staurosporin (1 µM) und Chelerythrin (10 µM) sowie Caspase-Inhibitoren Z-IETD-FMK (50 µM) und Z-DEVD-FMK (10 µM) hemmten die Camalexin induzierte Eryptose.Zusammenfassend zeigt die vorliegende Studie das Schrumpfen des Zellvolumens und die

Umlagerung von Phospholipiden auf der Plasmamembran nach Behandlung mit Camalexin. Dieser Effekt hängt zumindest teilweise von der Ca^{2+}-Rekrutierung sowie von bestimmten Caspasen und Kinasen ab.

8. LIST OF PUBLICATIONS

1. Almasry, Mustafa, Mohamed Jemaà, Morena Mischitelli, Caterina Faggio, and Florian Lang. "Stimulation of Suicidal Erythrocyte Death by Phosphatase Inhibitor Calyculin A." *Cellular Physiology and Biochemistry* 40.1-2 (2016): 163-71. Web.

2. Almasry, Mustafa, Mohamed Jemaà, Morena Mischitelli, Florian Lang, and Caterina Faggio. "Camalexin-Induced Cell Membrane Scrambling and Cell Shrinkage in Human Erythrocytes." *Cellular Physiology and Biochemistry* (2017): 731-41. Web.

3. Jemaà, Mohamed, Morena Mischitelli, Myriam Fezai, Mustafa Almasry, Caterina Faggio, and Florian Lang. "Stimulation of Suicidal Erythrocyte Death by the CDC25 Inhibitor NSC-95397." *Cellular Physiology and Biochemistry* 40.3-4 (2016): 597-607. Web.

4. Mischitelli, Morena, Mohamed Jemaàa, Myriamfezai Fezai, Mustafa Almasry, Florian Lang, and Caterina Faggio. "Stimulation of Erythrocyte Cell Membrane Scrambling by Adarotene." *Cellular Physiology and Biochemistry* (2017): 519-29. Web.

5. Mischitelli, Morena, Mohamed Jemaà, Mustafa Almasry, Caterina Faggio, and Florian Lang. "Triggering of Erythrocyte Cell Membrane Scrambling by Emodin." *Cellular Physiology and Biochemistry* 40.1-2 (2016): 91-103. Web.

6. Mischitelli, Morena, Mohamed Jemaà, Mustafa Almasry, Caterina Faggio, and Florian Lang. "Stimulation of Erythrocyte Cell Membrane Scrambling by Quinine." *Cellular Physiology and Biochemistry* 40.3-4 (2016): 657-67. Web.

7. Mischitelli, Morena, Mohamed Jemaà, Mustafa Almasry, Caterina Faggio, and Florian Lang. "Ca^{2} Entry, Oxidative Stress, Ceramide and Suicidal Erythrocyte Death Following Diosgenin Treatment." *Cellular Physiology and Biochemistry* 39.4 (2016): 1626-637. Web.

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9. Mischitelli, Morena, Mohamed Jemaà, Mustafa Almasry, Caterina Faggio, and Florian Lang. "Stimulation of Suicidal Erythrocyte Death by Rottlerin." *Cellular Physiology and Biochemistry* 40.3-4 (2016): 558-66. Web.

9. DISSERTATION STATENMENT AND CONTRIBUTIONS

The following dissertation was performed at the Institute of Physiology of the Eberhard-Karls-University Tübingen under the supervision of Professor Dr. Florian Lang. The study was designed in collaboration with Dr. Muhamed Jumaeh. Training was performed by the Masters student Miss Morena Mitschili. All experiments were performed independently and personally.

Figures of the statistical analysis were carried out by Dr. Muhamed Jumaeh after data collection and analysis. Statistical analysis was performed using two-way ANOVA with Tukey's post-hoc test, as indicated in the figure legends.

I hereby confirm that the manuscript associated with this thesis was prepared with the help of Tanja Loch. The study was supported by the Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Tübingen University under the supervision of Professor Florian Lang. Any additional sources of information have been duly cited.

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