The alkaloid narciclasine exerts anti-inflammatory actions by interfering with endothelial activation processes

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Dedicated to my always supportive family

Any accomplishment of mine would never be possible without you in my life

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Присвячується моїй завжди мене підтримуючій родині

Кожне моє досягнення не було б можливим без вас у моєму житті

TABLE OF CONTENTS

1 INTRODUCTION

1.1   The vascular endothelium

The vascular endothelium represents a layer of endothelial cells that are bound together and build the inner lining of the blood vessels. The word "endothelium" was used in 1885 by Swiss anatomist Wilhelm His for the first time.[1] However, only a hundred years later did vascular endothelium become an object of active research. One of the first studies on the morphology of the endothelium, different types of endothelial cells, endothelial junctions and their behavior was done by Florey and colleagues.[2] This marked the beginning of further extensive studies on the endothelium and its main functions.

The endothelium is one cell layer thick in all vessels, and in capillaries it comprises the only cell layer of the vessel wall.[3] Its structure within blood vessels is shown in
Figure 1.
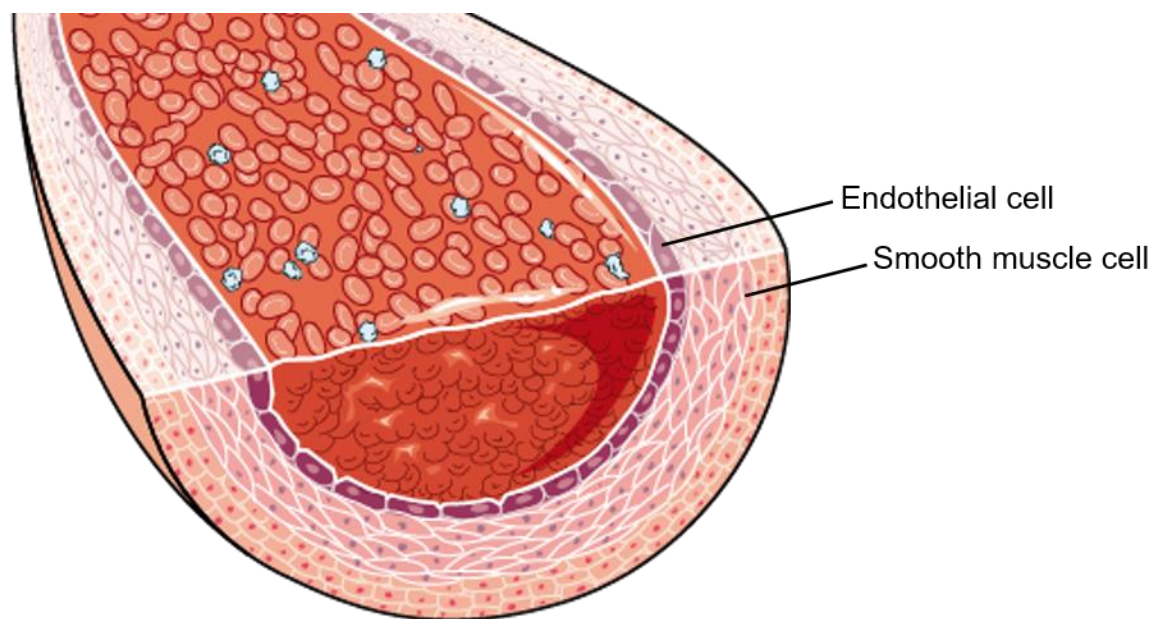
Figure 1: Structure of the vascular endothelium.
The endothelium covers the inner lining of the blood vessels and capillaries with one layer of cells. Depending on the type of a vessel, the endothelial cell layer can be surrounded by one or multiple layers of smooth muscle cells. This picture representing the structure of the blood vessel was taken from an online repository https://smart.servier.com/.

The endothelium is an essential part of the vascular system - its coverage of the inner lining of all blood vessels accounts for an area of 7 m2.[4] Because of this and the ability of endothelial cells to interact with their environment and with each other, the vascular endothelium executes many roles withing a living organism.[5, 6] The

endothelium has endocrine properties[7] and functions as a receptor-effector organ. The receptor-effector function is executed by the synthesis and release of necessary proteins or by modifying the shape of the vessel as a reaction to different physical and chemical stimuli. This contributes to the maintenance of homeostasis and the integrity of the blood vessels. The endothelium can produce agonists and antagonists for different processes depending on the current needs such as, vasoconstrictors and vasodilators, pro- and anticoagulants, substances with oxidizing and antioxidizing effects, pro- and anti-inflammatory cytokines.[8-10] The main functions of the vascular endothelium are the formation of new blood vessels and the remodeling of existing ones, regulation of vascular tone, regulation of blood clotting, barrier function and working together with the immune system in reaction to the inflammation caused by infection.[5, 11, 12] The endothelium executes its main functions by secreting several active substances **(Table 1).**[6, 13]

Table 1: Subtances, which are produced by endothelial cells to maintain homeostasis[6, 13]

Function / Other products	Secreted substance
Vasodilation	Nitric oxide, prostacyclin, endothelium derived hyperpolarizing factor
Vasoconstriction	Angiotensin converting enzyme, thromboxane A2, leukotrienes, free radicals, endothelin
Vasculogenesis / Angiogenesis	Vascular endothelial growth factor 1, 2
Coagulation	Von Willebrand factor, thromboxane A2, factor V, thromboplastin, platelet activating factor, plasminogen activator inhibitor
Antithrombotic function	Prostacyclin, thrombomodulin, antithrombin, heparin, plasminogen activator
Inflammation	Leukotrienes, MHC II, interleukins 1, 6, 8
Growth factors	Insulin-like growth factor, transforming growth factor, colony stimulating factor
Lipid metabolism	LDL-receptor, lipoprotein lipase
Matrix Products	Fibronectin, laminin, collagen, proteoglycans, proteases

Dysfunction of the endothelium may result in the development of severe conditions, such as atherosclerosis, thrombosis, acute ischemic syndrome, diabetes and autoimmune inflammatory disorders.[11, 14-17]

1.1.1   Phenotypical differences of the endothelium in the different parts of the human body

The endothelium has a different structure depending on its localization in the body.[12] The phenotypical differences of the endothelium between organs are bound to the function of the endothelial cells in the specific organ or tissue. For example, endothelial cells in the blood vessels that spread through the liver lack a typical basement membrane and contain open pores.[18] This structure supports an important liver clearance function and the metabolism of macromolecules (glycoproteins, lipoproteins, extracellular matrix components, inert colloids).[19, 20] Another example of the special structure of the endothelium is the blood-brain barrier (BBB). In the BBB, endothelial cells are held together stronger by the tight junctions than in any other system of the human body.[21] In addition, endothelial cells of the BBB contain less vesicles, which facilitates the transport of molecules.[22] This helps to maintain intactness of the brain and high selectivity of the molecules that are transferred to the brain. These differences in the phenotype allow endothelial cells to execute their physiological functions depending on the needs of the specific underlying tissue.

1.1.2   Role of the endothelium for vascular permeability

Endothelial cells play an important role in vascular permeability. Vascular permeability enables the exchange of active hormones, molecules and immune cells between the blood stream and organ tissues. Small molecules and hormones can travel directly through *fenestrae*, which represent pores between endothelial cells. Bigger molecules can go through paracellular junctions, which are located between endothelial cells. Finally, cells like leukocytes can transmigrate through the endothelial cell layer.[23] In physiological conditions, vascular permeability is important for active substances to get from the blood stream to the tissue and to enable the immune response.

The connectivity of endothelial cells is regulated by different kinds of junctions, which are formed between the cells. These junctions are adherence junctions, tight junctions and gap junctions.[23] Tight junctions regulate the connectivity of the endothelial cells and stabilize their barrier function. They are present in the

endothelial cells, which form arteries and veins throughout the human body. Tight junctions are particularly important for the endothelial cells that form blood-brain-barrier (BBB).[24] They stabilize the BBB layer and contribute to its integrity. Gap junctions are utilized when signaling molecules, ions, electrical current and transmembrane potential need to be transferred directly from one cell to another.[25] Adherence junctions are the main junctions responsible for the EC barrier function. The main components of these junctions belong to the cadherin family. VE-cadherin is a special cadherin found in endothelial cells. Together with other cadherins (N-cadherin, P-cadherin, T-cadherin) VE-cadherin participates in the remodeling of the cytoskeleton to form adherence junctions.[26, 27] Among other components involved in the formation of adherence junctions are catenins (α-catenin, β-catenin, γ-catenin) and p120, a binding partner of VE-cadherin.[28-31] Adherence junctions can be found in blood and lymphatic vessels where they play an important role in the permeability of endothelial cells for leukocyte migration during the inflammatory process.[27]

1.2 Inflammation
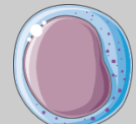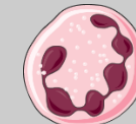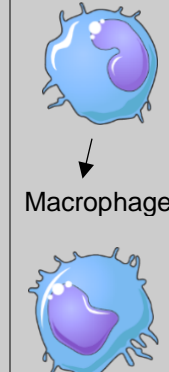
Inflammation is an immune response in reaction to the injury of the tissue or to pathogens in the living organism. Any bacteria, virus, fungi or parasites are recognized as pathogens and are successfully eliminated by the immune system. During an injury inflammation promotes wound healing and helps to prevent infection of the whole body. In these cases, inflammation plays a beneficial role and helps to maintain the physiological homeostasis of the organism.[32-35]

However, it has been recognized that chronic inflammation can develop without any external stimulation. Organism's own organs, tissues and cells can be perceived as hostile targets and the immune system reacts with the development of a non-physiological autoimmune inflammation.[36, 37] It is considered that chronic inflammation is a cause of diseases such as chronic kidney inflammation, rheumatoid arthritis, atherosclerosis, multiple sclerosis and Alzheimer's disease.[37-40]

The key cellular players in the inflammatory response are leukocytes, which are circulating in the blood stream, and endothelial cells, which are located on the inner lining of the blood vessel.[41]

Leukocytes are derived from lymphoid and myeloid stem cell progenitors **(Table 2)**. Leukocytes with myeloid stem cell progenitors play a role in the innate immunity (basophiles, eosinophils, neutrophils, monocytes). Lymphoid stem cell progenitors generate B- cells, T-cells and natural killer (NK) cells, which contribute to the development of adaptive immunity. B-lymphocytes offer a specific response with their ability to produce antibodies specific to pathogens.[42] Whereas T-lymphocytes can directly interact with pathogens and eliminate them (cytotoxic T-cells) or contribute to immunity by influencing how other cells of the immune system interact with the pathogen (helper T-cells).[43]

Table 2. Leukocytes derived from lymphoid and myeloid stem cell progenitors. Pictures representing cells were taken from the online repository https://smart.servier.com/

Hematopoietic stem cells						
Pluripotent stem cells						
Lymphoid stem cells			Myeloid stem cells			
B lympho-cyte	T lympho-cyte	NK Cell	Basophil granulo-cyte	Eosinophil granulo-cyte	Neutrophil granulocyte	Monocyte
						Macrophage

Endothelial cells get activated by the cytokines, which are expressed by leukocytes. Activated EC facilitate migration of the leukocytes through the endothelial barrier of the blood vessel. The interaction between these types of cells allows leukocytes to leave the bloodstream, enter the tissue of the organs and start the immune response.[44, 45]

1.2.1 The interaction between leukocytes and endothelial cells during inflammation development

Inflammation development is dependent on the complex mechanism of the interaction between leukocytes and endothelial cells (ECs).[46] Leukocytes secrete inflammatory factors, which activate endothelial cells. In return, activated ECs facilitate migration of the leukocytes through the endothelial cell layer of the blood vessel **(Figure 2)**. To launch this process, leukocytes first need be tethered to the endothelial cells. This happens due to binding of L-selectin and very late antigen (VLA-4), which are located on the leukocyte surface, to sialyl-Lewis$_x$ carbohydrate antigen (s-Le$_x$) and vascular cell adhesion molecule-1 (VCAM-1) on EC, respectively.[47] Tethered leukocytes start rolling on the surface of the endothelium.
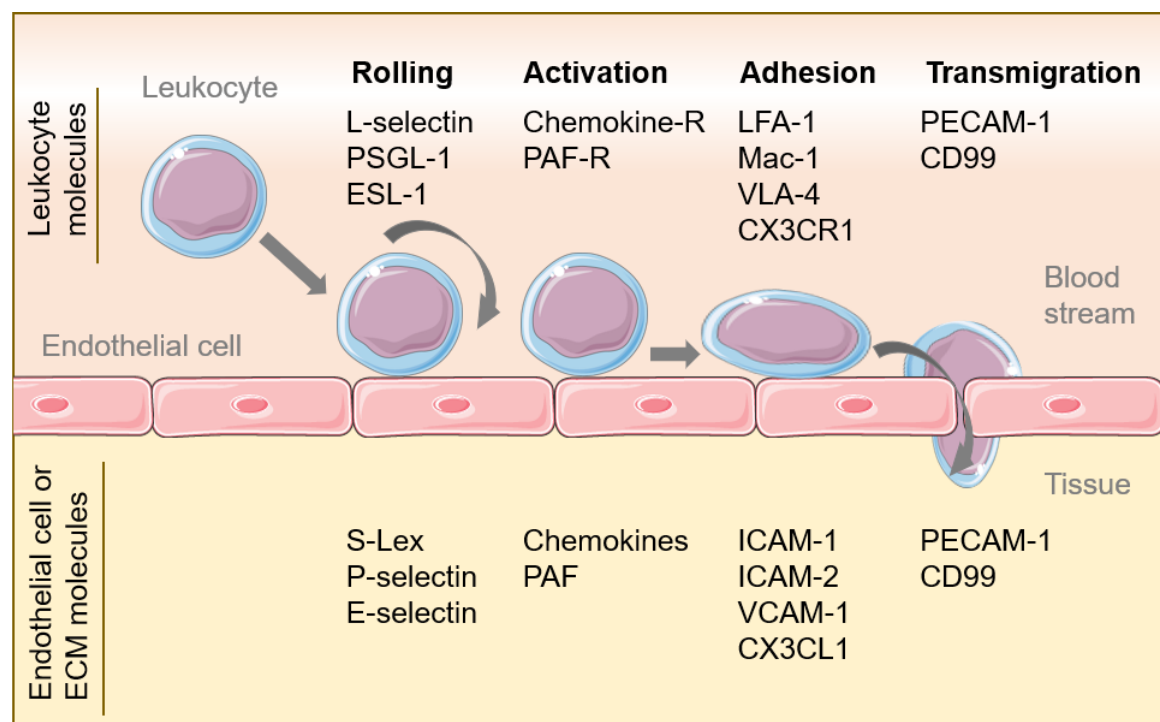
Figure 2: Leukocyte adhesion and transmigration through the endothelium.
Leukocytes start to roll on the surface of the endothelium. They express L-selectin, PSGL-1 and ESL-1 which bind to their corresponding ligands s-Le$_x$, P- and E-selectin on the endothelial cell surface. Leukocytes get activated with help of the signals coming from EC. During the adhesion process molecules expressed on leukocytes LFA-1, MAC-1, VLA-4 and CX3CR1 bind to their ligands on the EC: ICAM-1, ICAM-2, VCAM-1 and CX3CL1. PECAM-1 and CD99 located on both leukocytes and EC facilitates the transmigration of the leukocytes through the endothelial cell layer to the site where they cause inflammation. Figure adapted from Muller, 2002.[47] Pictures representing cells were taken from the online repository https://smart.servier.com/

Rolling of leukocytes is mediated by endothelial-leukocyte adhesion molecule 1 (ELAM-1, E-selectin) and P-selectin and s-Le$_x$ expressed on the EC.[48] The expression of E-selectin can be triggered on the surface of the endothelial cells by different cytokines, such as IL-1α, IL-1β, tumor necrosis factor α (TNFα) or endotoxins.[49] Rolling is followed by the activation of leukocytes which is facilitated by the signals coming from endothelial cells *via* G protein-coupled receptors.[47]

During the next steps, leukocyte integrins recognize intracellular cell adhesion molecule 1 and 2 (ICAM-1, ICAM-2) and vascular cell adhesion molecule 1 (VCAM-1) as their ligands and bind to them.[46] These molecules facilitate the adhesion of the leukocytes by weakening the junctions between endothelial cells. The mode of action of ICAM-1 is through the phosphorylation of VE-cadherin, which stimulates the disassembling of adherence junctions.[50] VCAM-1 activates the small GTPase Rac 1.[51] The activation of Rac 1 increases the production of reactive oxygen species (ROS). The action of both ICAM-1 and VCAM-1 results in the loosening of adherence junctions. Leukocytes transmigrate through the holes that formed where the adherence junction used to be. With the help of the interaction between PECAM-1 and CD99 located on both leukocytes and endothelial cells, adhered leukocytes transmigrate through the endothelial cell layer to the tissue of the organs and contribute to inflammation.[47, 52]

Among the variety of molecules and chemokines, which take part in the interaction between EC and leukocytes, recently one more player has been recognized – the chemokine fractalkine (CX3CL1).[53] Fractalkine contains a unique CX3C motif. It is expressed by endothelial cells as a reaction to a stimulation by IL-1β, TNFα, interferon-γ or lipopolysaccharide (LPS).[53, 54] Fractalkine is expressed by ECs in two forms: membrane-bond and soluble. The membrane-bond form of CX3CL1 acts as an adhesion molecule for monocytes and lymphocytes, which have a receptor to the fractalkine on their surface (CX3CR1).[55] The soluble form of fractalkine is a chemotactic agent.[56] In the process of interaction between leukocytes and endothelium, fractalkine binds to its corresponding receptor CX3CR1 on the EC and represents one more molecule that promotes adhesion of the leukocytes to the endothelium.[57]

As endothelial cells play a direct role in the development of inflammation, targeting the interaction between leukocytes and ECs has been recognized as promising approach to reduce the development of chronic inflammation.[58, 59]

To understand which part of the inflammation process can be targeted, in the following part of this introduction the molecular mechanisms that are involved in the development of inflammation will be presented.

1.2.2 Molecular mechanisms involved in the inflammation development

Depending on the signaling molecule that triggers inflammation, different pathways are activated in the cell. Among the most common triggers of inflammation are IL-1α, IL-1β, IL-6, TNFα, interferon-γ, LPS.[34, 41] When activated, monocytes and neutrophils release cytokines that trigger the production of prostaglandins (PGs). PGs contribute to the inflammation by, for example, increasing arterial dilation and vascular permeability thus increasing a blood flow to the inflamed tissue.[60]

The part of the inflammatory response that requires new protein synthesis is regulated by molecular pathways within the cell. One of the pathways that contributes to inflammation development is the NF-κB pathway. Its canonical part can be activated by IL-1α, TNFα, lipopolysaccharide (LPS), reactive oxygen species (ROS) and isoproterenol.[61, 62] When this pathway is triggered by IL-1α and TNFα, these inflammatory stimuli need to first bind to their corresponding receptor on the endothelial cell surface. This binding further triggers the phosphorylation cascade for TAK-1, IKKβ and IκBα in the cell cytoplasm. IκBα is bound to NF-κB transcription factor. When IκBα gets phosphorylated, it is released from NF-κB. Free NF-κB translocates into the nucleus, where it is involved in the regulation of the genes important for the development of inflammation.[61]

Another pathway that partly contributes to the development of inflammation on the molecular level is the mitogen-activated protein kinase (MAPK) pathway. It consists of multiple steps of phosphorylation, which sequentially activate different kinases. As a result of a complex phosphorylation cascade, target proteins get

activated and contribute, among other processes, to inflammation development.[63, 64]

1.2.2.1   Nuclear factor NF-κB pathway in inflammation

NF-κB pathway can be divided into a canonical and alternative pathway **(Error! Reference source not found.)**.[61] The canonical pathway is activated by IL-1α and TNFα directly contributes to the development of inflammation. These stimulation factors bind to their receptors on the cell surface. Through a cascade involving TRAF and TAB proteins, TAK-1 kinase gets phosphorylated.[65] TAK-1 phosphorylates IKKβ kinase, which is located in the IKK complex. The IKK complex is considered to be the main trigger for NF-κB pathway activation.[66] It consists of three subunits: IKKα, IKKβ, and NEMO/IKKγ. While the exact role of NEMO remains unclear, it is known that IKKβ phosphorylates IκBα. IκBα is a part of the NF-κB complex, located within the cell cytoplasm. This complex consists of a IκBα subunit, p65/RelA and p50/NF-κB subunits. The IκBα subunit keeps the NF-κB transcription factor inactive in the cytoplasm and does not allow its translocation into the nucleus.[67] When IκBα gets phosphorylated by IKKβ, it is released from the complex. Which allows Rel/NF-κB to translocate into the nucleus.

NF-κB transcription factor regulates the expression of a variety of genes in the nucleus. Proteins that are being synthesized from genes regulated by NF-κB are important for survival, proliferation, inflammation and immune regulation.[61, 68] In particular, NF-κB regulates the expression of genes coding for cell adhesion molecules, which, after protein synthesis, are expressed on the surface of the endothelial cells.[69] The cell adhesion molecules facilitate leukocytes rolling and their adhesion to endothelial cells.[48, 52] These processes are crucial for the development of inflammation.

The alternative NF-κB pathway is activated by LT and CD40L through B cell activating factor (BAFF). IKKα phosphorylates p100, leading to the activation of p52/RelB heterodimers.[61] The alternative NF-κB pathway is important for lymph-organogenesis and B-cell activation.[70, 71] Its role in the development of inflammation has not been identified yet.

Targeting of the canonical NF-κB pathway has been recognized as an effective approach in reducing inflammation.[72, 73] A number of drugs containing active substances, which block NF-κB pathway as their mechanisms of action has been developed. Glucocorticoids, aspirin and sodium salicylate are among them.[74, 75]
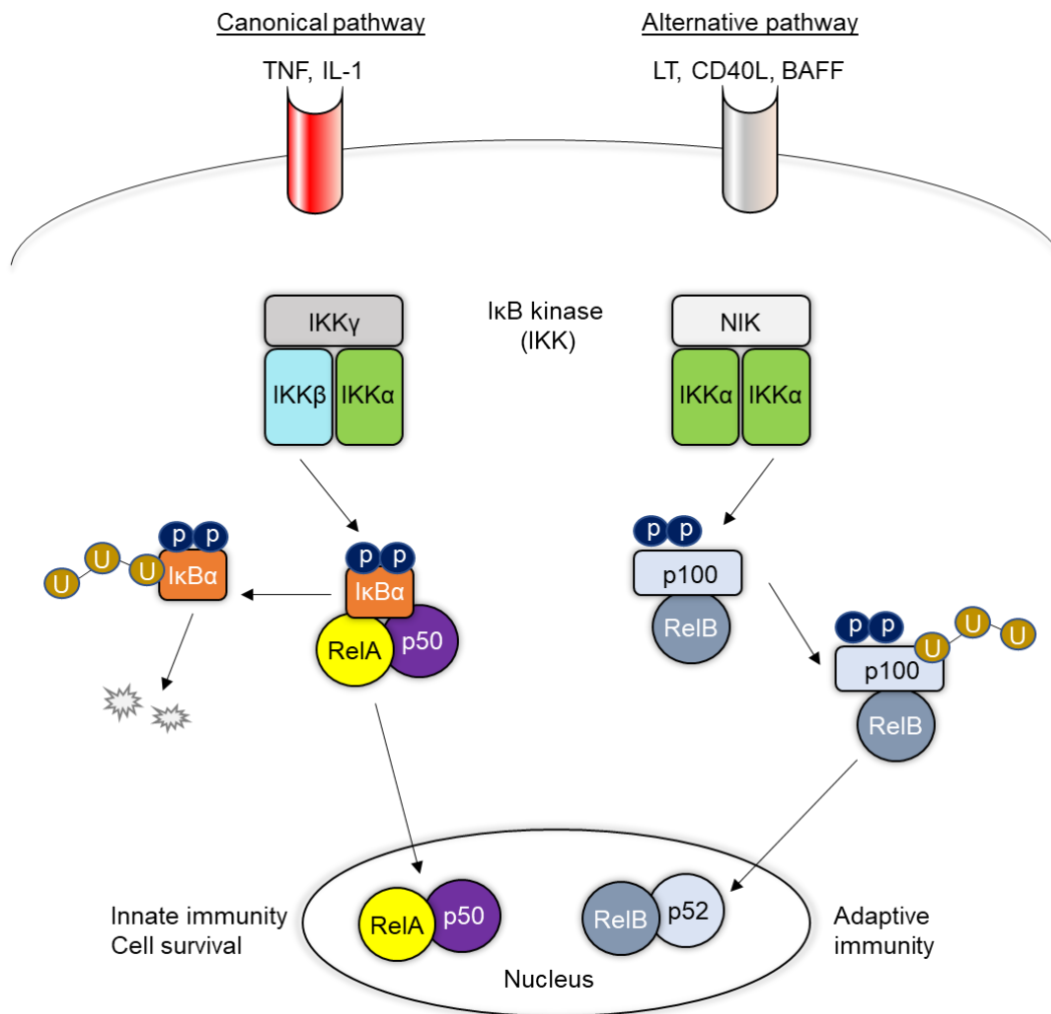
Figure 3: NF-κB pathway.
The activation of the canonical pathway happens after the stimulation of the endothelial cells with IL-1 and TNF. The IKKβ from the IKK complex phosphorylate IκBα. Phosphorylated IκBα detaches from the RelA/p50 (NF-κB) complex. RelA/p50 (NF-κB) translocates into the nucleus and triggers the expressions of the genes important for cell survival and inflammation development. The alternative pathway is triggered by LT and CD40L through BAFF. IKKα from the IKK complex phosphorylates p100, bound to RelB. After the processing of p100 it turns into p52. The RelB/p52 complex translocates into the nucleus and is responsible for activating genes important for lymph-organogenesis and B-cell activation. Based on Lawrence *et al*, 2009.[61]

1.2.2.2 Mitogen-activated protein kinase (MAPK) pathway in inflammation and the role of MKP-1

The mitogen-activated protein kinase (MAPK) pathway contributes to a variety of processes connected to cell regulation. Among them are gene transcription activation, cell death, protein synthesis and cell differentiation. The MAPK pathway is activated through tyrosine kinases receptors. It can be trigged in mammalian cells through TNF, TGF-β, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin. To become active, each MAP kinase requires an activation by an upstream MAP kinase (MAPKK) and this MAPKK needs to be also activated by the corresponding upstream kinase (MAPKKK).[76] The whole cascade leads to the phosphorylation of kinases, which, at the end, though transcription factors activate target proteins **(Figure 4)**.[76]
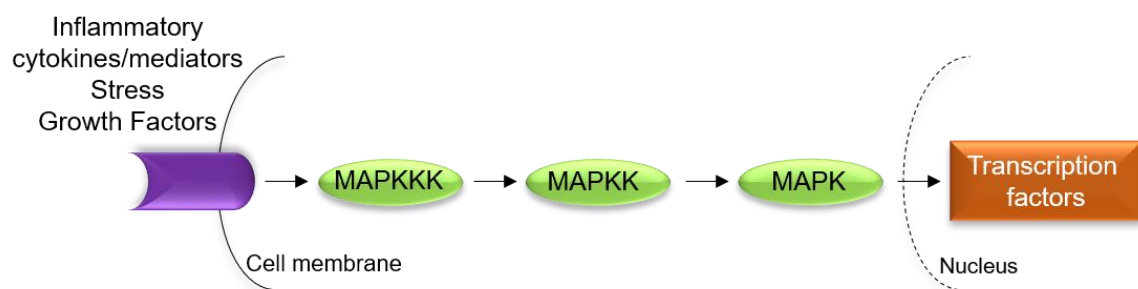
Figure 4: Activation of MAP kinases in the MAPK pathway.
After binding of inflammatory cytokines/mediators, the signal from the receptor activates MAPK kinase kinase (MAPKKK). Following the activation cascade MAP kinase (MAPK) gets phosphorylated and activates transcription factors in the nucleus.

In mammalian cells there are three main classes of MAP kinases: two stress-activated protein kinase (SAPKs) families and the extracellular signal-regulated kinases (ERKs). The two SAPKs families are the c-jun N-terminal kinase (JNK) and p38.[77] JNK and p38 kinases contribute to inflammation development through MAPK pathway. When activated, p38 induces the expression of VCAM-1, which contributes to adherence of the leukocytes to the endothelium.[63] As a response to the cytokines stimulation, JNK kinase activates transcription factor activation protein 1 (AP1).[78] Among other genes, AP-1 is responsible for the expression of E-selectin, which helps leukocytes to adhere to the endothelium.[79, 80]

The negative feedback to the activation of MAP kinases is executed in the cell using dual specificity phosphatases (DUSPs), tyrosine phosphatases and threonine phosphatases.[81] The inhibition of p38 and JNK activation is executed by a dual specificity phosphatase – 1 (DUSP-1 or MKP-1) **(Figure 5)**.[82]
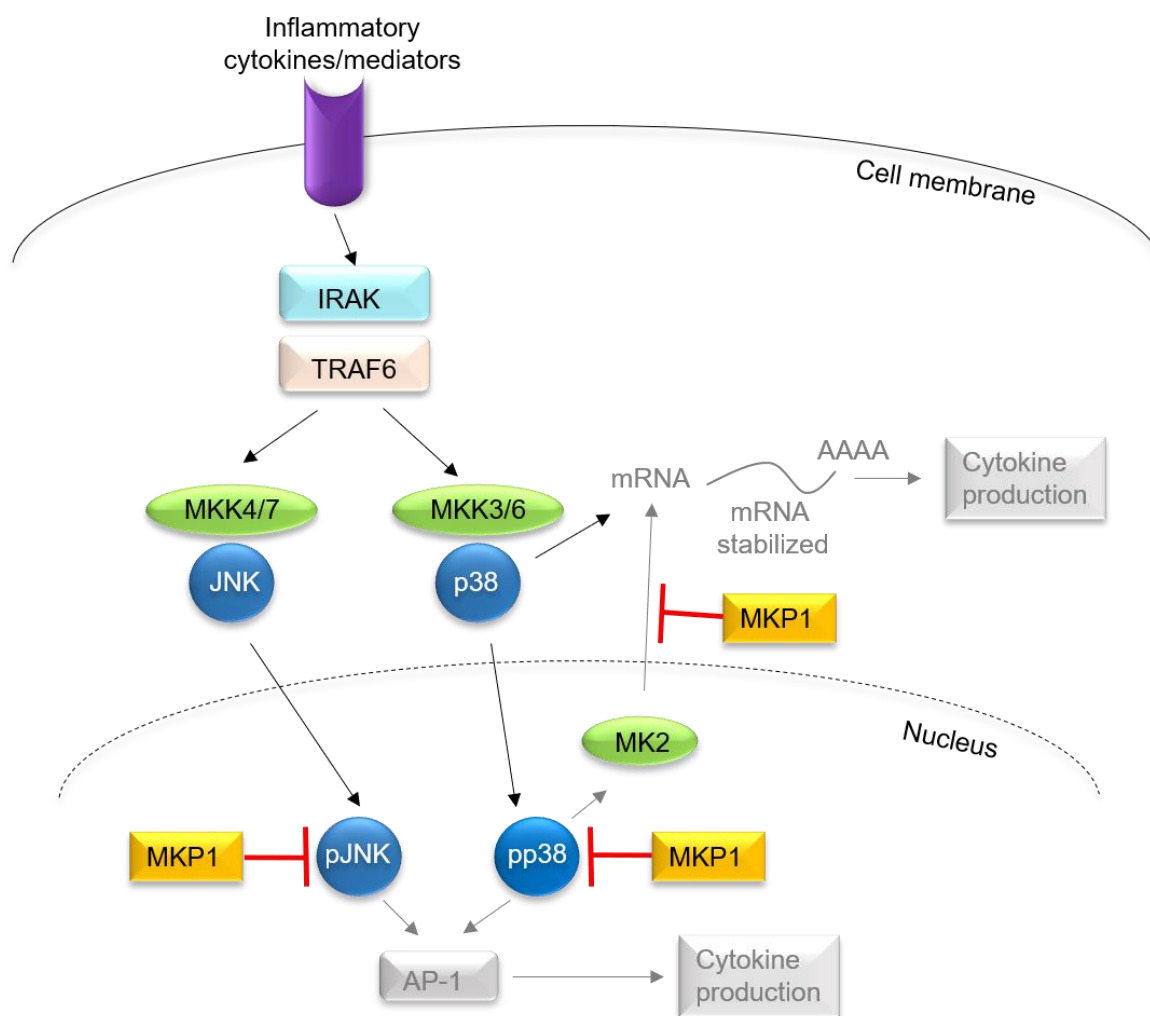
Figure 5: Regulation of MAPK pathway by MKP-1.
Inflammatory cytokines and mediators bind to their corresponding receptors on the cell surface. This binding recruits common upstream activator IRAK/TRAF6. Following the MAPK activation cascade, phosphorylated kinases p38 and JNK move to the nucleus where they bind to the AP-1 motif in the promoter of target genes. This induces the transcription of genes which lead subsequently to cytokine production. MKP-1 has an ability to negatively regulate the phosphorylation of JNK and p38. This prevents AP-1-dependent cytokine production. The figure was adapted from 2012 Qiyan Li et al.[82]

MKP-1 protein expression can be induced by LPS, heat shock, hypoxia, dexamethasone or oxidative stress.[83] MKP-1 prevents cells from undergoing

apoptosis and suppresses pro-inflammatory cytokine production in macrophages by reducing the phosphorylation and, thus, the activation of p38 and JNK.[84]

Targeting of p38 and JNK has shown to be beneficial in both *in vivo* and *in vitro* research of arthritis and inflammation.[85] The positive effect in reduction of paw swelling, cartilage breakdown, inflammation and bone erosion was demonstrated after the application of p38 inhibitors in the CIA mice,[86] adjuvant and CIA rat models[87, 88] and rat streptococcal cell wall arthritis model.[89, 90] In *in vitro* experiments, inhibition of p38 successfully blocked IL-1, IL-8, TNF, and cyclooxygenase-2 (COX2) production in monocytes. The research with specific JNK inhibitors showed that JNK plays a role in the regulation of production of collagenase by fibroblasts form synovial fluid.[91]

1.3 Natural compounds in drug discovery

Natural compounds are substances and small molecules, which are produced by or being isolated from microorganisms, plants, marine organisms and animals. Compounds produced by living organisms can be divided into primary and secondary metabolites. Primary metabolites are synthesized to support normal growth and development of the organism. Secondary metabolites are produced as a reaction to changing environmental conditions or threats coming from other living organisms. They can be classified to terpenoids, steroids, alkaloids, fatty acid-derived substances, polyketides, non-ribosomal polypeptides and enzyme cofactors.[92] Secondary metabolites are the source of unique bioactive natural compounds and have successfully been used in development of different drugs. After synthesis and characterization some natural compounds are used directly as components of a drug and some need to be modified before they become a part of a medicinal product.

Among natural products derived from animal, plants or microorganisms, the plant-derived natural compounds have been used for medicinal purpose the longest. There are findings reporting the use of plants to treat a variety of diseases since 2900 BC.[93] Of course, at that time no exact active substance could be isolated. People used simple water extracts, mashed leaves of the plants to cover wounds or prepared tea from herbs. The techniques for extraction and purification of active

components from the plants have improved throughout the human history. Plants became a source for many active substances which are a base for many drugs used today. For example, quinine and artemisinin were derived from plants and are used in drugs against malaria. These compounds originate from *Cinchona spp.* (quinine) *and Artemisia annua* (artemisinin).[94-96] Anticancer drugs paclitaxel and vinblastine are also of plant origin,[97, 98] as well as morphine, which was obtained from the plant *Papaver somniferum*.[99]

Besides plants, microorganisms are an important source of bioactive natural products. The isolation of penicillin produced by the fungus *Penicillium notatum* marked the beginning of the antibiotics era.[100] This discovery has completely changed human history by opening a way to treat bacterial-caused diseases that were once the leading cause of death and forever reframed the landscape for drug discovery.[101] From that point on microorganisms became a valuable source of a variety of active substances and constant objects for screening and scientific research.[102-104] Microorganisms are not only a source for antibacterial products but also products which are effective against parasites (e.g. ivermectin from *Streptomyces* species).[105] *Streptomyces* species produce immunosuppressive agents cyclosporin and rapamycin.[106] Mevastatin and lovastatin are known to lower cholesterol levels and are also produced by *Penicillium* species.[107, 108]

Bioactive natural compounds of marine origin have not been fully explored so far. There are several products derived from marine organisms, which are currently in different phases of clinical trials. Currently ongoing clinical trials aimed at different kinds of cancer are being performed with ecteinascidin 743, spisulosine and kahalalide F, which were isolated from marine organisms *Ecteinascidia turbinate, Spisula polynyma* and *Elysia rufescens* respectively.[109-111]

Without a doubt, natural products have become a base for many drugs used in modern medicine. Nowadays the manufacturing of medicine based on natural compounds represent around a quarter of all drugs approved by governmental authorities Food and Drug Administration (FDA) and/or the European Medical Agency (EMA).[112] As until now less than 10% of biodiversity has been screened as a potential source of bioactive compounds,[113] the search for further bioactive substances will continue.

1.3.1 Obtaining of bioactive natural compounds from plants

Plants can be used in manufacturing of modern pharmaceuticals in the form of an extract or in the form of a pure bioactive compound, which is isolated from the extract. Before starting the extraction and purification of a natural product, it is important to make sure that the plant species had been chosen correctly. Together with morphological identification, genomics helps to identify species based on DNA markers – short DNA sequences specific for certain species.[114, 115]

Extraction and purification of the active substance from the plants became an important part of drug screening and further manufacturing of pharmaceuticals. The basic process includes selection of the material, extraction and separation of the active substances, elucidation of the chemical structure and bioactivity and toxicology research **(**

Figure *6*).[116]

Selection of the plant material is based on the data already available in scientific literature, information from the ethnomedicine, phytochemistry and taxonomy of the plant. When plant material is selected, its extract is divided into separate fractions. These fractions undergo bioassays to elucidate if they have any beneficial biological activity. Pure compounds are separated from the fractions, which demonstrated the most promising effects. The structure of these compounds can be modified and/or synthesized as is. Further biological and toxicological investigation is then needed to confirm bioactivity of the isolated compounds.[116]

Despite a great success in the purification of extracts and identification of the bioactive compounds, which are used as a base for manufacturing of drugs, there are a number of limitations connected to the therapeutic efficacy of plant-based medicine.[117] Completely plant-based products, which include full extracts from the plants, seldomly undergo controlled and standardized clinical trials. Taken together, the complication of drug discovery is sometimes based on the synergetic action of two or more active substances within the plant.[118, 119] Thus, even if an extract shows beneficial effects, the identification of the exact combination of substances, which contribute to this beneficial effect takes a lot of time and effort.
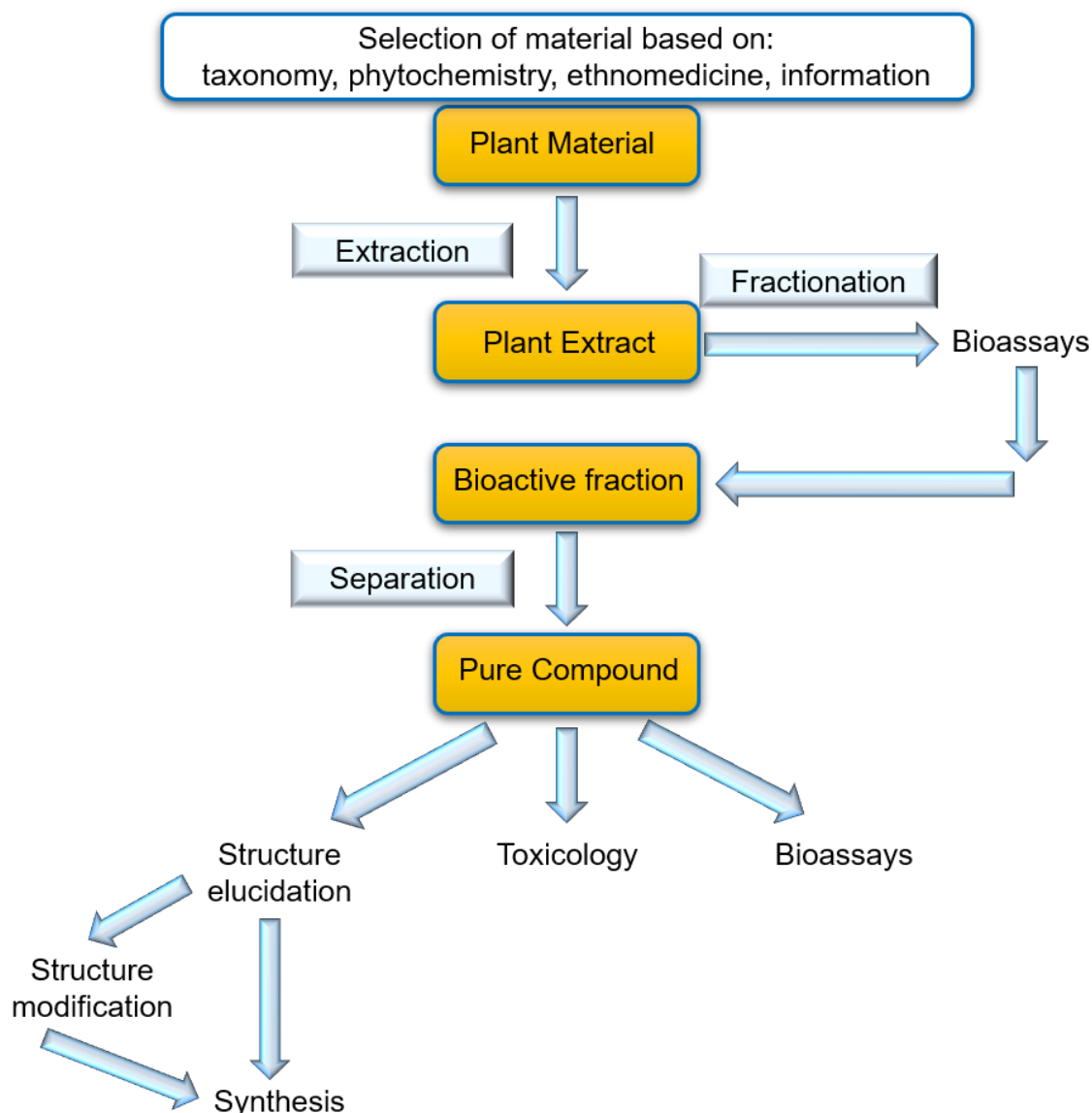
Figure 6: Identification of the bioactive components from plants.
Material is being selected based on the following approaches: taxonomic, phytochemical, ethnomedical, analysis of available information in the literature. After obtaining an extract from the plant material, fractionation of this extract is performed. Fractions of the extract are tested in bioassays. Pure compound is separated from the fractions that showed positive effect in bioassays. After structure elucidation, pure compound is modified or synthesized directly. This compound is used in bioassays to investigate its properties in more details. This figure is adapted from Koparde *et.al*, 2019. [116]

In cases where the bioactive compounds were identified and isolated, it is not always possible to elucidate the molecular mechanisms of their action even if the biological effect is very prominent. The comparison of a structure from newly

synthesized compounds to already existing ones can help to make an assumption about proteins that this new compound might be targeting. There are online databases that contain information about structure of known active compounds and their target proteins and pathways (CheEMBL, NIH Small Molecules Repository, UniProt).[120-122] Similarity in structure does not always mean that the compounds will have the same biological activity, but it does give an idea which proteins should be investigated at the beginning of the research.

In future of plant-based natural products will continue to be a valuable source of bioactive compounds. Nevertheless, to keep up with the growing need for new effective drugs, the screening and further research will need to happen faster than it is currently. To enable this, new profiling technologies and computational techniques, which include big data analysis and artificial intelligence, will need to be further developed and applied for plant-based natural compounds screening and research.[123]

1.3.2 Narciclasine

Narciclasine or lycoricidinol is an isocarbostyril alkaloid which can be found in different *Narcissus species* (Amaryllidaceae). The plants of the genus *Narcissus* have been used to treat cancer-like diseases since ca 460-370 B.C. in a form of an oil.[124] As pure compound narciclasine was first isolated in 1967.[125] However, it took another five years for the chemical structure to be fully characterized **(Figure 7)**.[126]
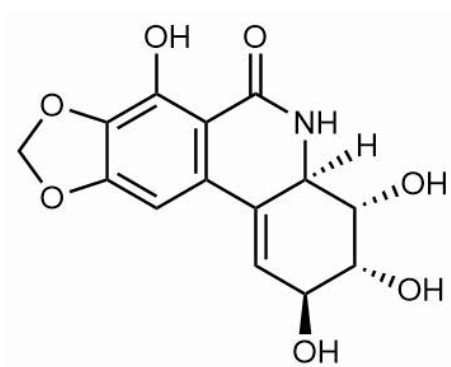
Figure 7: Chemical structure of the isocarbostyril alkaloid narciclasine.

One of the first studies performed with narciclasine revealed that it interacts with the 60S ribosome subunit and inhibits the peptide bond formation by eukaryotic ribosomes[127]. This study was followed by the discovery that narciclasine could inhibit protein translation.[128]

Since its first synthesis and structural characterization narciclasine has been extensively studied as a potential drug against different kinds of cancer.[129-131] *In vitro* research on glioblastoma showed that narciclasine exerts profound proapoptotic and cytotoxic activity against cancer cells in the concentration of approximately 1μM. In *in vivo* studies narciclasine showed beneficial effects on mice affected with glioblastoma when it was applied at a dose 10 mg/kg.[129] The mechanism explaining an anti-glioblastomal action of narciclasine is connected to the activation of the Rho stress fibers, which, when activated, increase focal adhesion.[132] Another evidence demonstrates that narciclasine activates Rho kinase in RhoA-independent manner.[133]

One more mechanism, which supports narciclasine's anti-cancer properties, is the targeting of the process of angiogenesis. There is an evidence that narciclasine influences angiogenesis by downregulating vascular endothelial growth factor receptor 2 (VEGF receptor 2) in endothelial cells.[133]

Narciclasine has shown promising results in melanoma research. It has demonstrated a beneficial effect in an apoptosis-resistant brain metastatic melanoma orthotopic model in immunocompromised mice. During this research the eukaryotic translation elongation factor 1 (eEF1A) was discovered to be narciclasine's target in cancer cells.[131]

Narciclasine inhibited the proliferation of triple-negative breast cancer cells due to the stimulation of autophagy-dependent apoptosis in breast cancer studies. This action was dependent on the regulation of AMPK-ULK1 (AMP-activated protein kinase - Unc-51 like autophagy activated kinase 1) signaling by narciclasine.[134]

Among further mechanisms explaining narciclasine's anticancer activity is its ability to induce apoptosis specifically on cancer cells. Dumont *et al.* showed that

narciclasine activates cell death receptor in cancer cells, which leads to apoptosis.[135]

Together with the profound study of narciclasine's anticancer activity, it has been recently recognized that the substance also has anti-inflammatory properties.[136] The first study that demonstrated this activity of narciclasine was published by Mikami *et al.* To perform this study *in vivo* rat adjuvant arthritis model was used. Narciclasine reduced cytotoxic effect of calprotectin, which is produced by neutrophils, and thus suppressed the development of arthritis in a rats.[137] In addition, one more study showing narciclasine's beneficial action against arthritis was done by Lubahn and colleagues. Sodium narcistatin, a cyclic phosphate prodrug of narciclasine, reduced inflammation and joint destruction in a rat adjuvant arthritis model.[138]

Narciclasine was also effective in an *in vitro* experimental model of Alzheimer's disease. Here, the major component of senile plaques formed during the disease neurotoxic amyloid-β was influenced by narciclasine. Narciclasine in the concentration range between 0.037 and 3.7 µM successfully reduced the levels of neurotoxic amyloid-β peptides in a dose-dependent manner.[139] This research was done in HeLa cells, which were preciously transfected with an amyloid precursor protein carrying the Swedish mutation up to 45 %. No cytotoxic effect of narciclasine on HeLa cells was observed.

The first findings in explanation of the anti-inflammatory effects of narciclasine demonstrated that the compound inhibits TNF-α and nitric oxide (NO) production by macrophages.[140, 141]

Research done in our working group by Dr. Simone Fuchs revealed the effect of *Haemanthus coccineus* extract (HCE) and its main bioactive component narciclasine on endothelial cells for the first time.[142] *In vivo* experiments demonstrated that the extract reduced ear edema formation and neutrophil infiltration in the acute and local inflammation model and attenuated macrophage extravasation in the murine unilateral ureteral obstruction model. *In vitro*, HCE blocked the release of TNFα, IL-6 and IL-1β by the leukocytes. The action of HCE on endothelial cells resulted in the reduction of leukocyte adhesion to the surface of the endothelial cells. The expression of the endothelial cell adhesion molecules ICAM-1, VCAM-1 and E-selectin was downregulated by the extract. Narciclasine

has been identified as a substance that was responsible for extract's activity. Further assays performed with narciclasine alone revealed that it could significantly reduce the expression of ICAM-1 on the surface of the endothelial cells.[142]

Considering the profound effects of narciclasine in the *in vivo* inflammation models and its activity under low concentration, there is a need for further investigation of the molecular mechanisms, responsible for this anti-inflammatory activity.

1.4 Aim of the study

Narciclasine is an alkaloid derived from plants of different *Narcissus* species.[125] During last two decades there were studies reporting narciclasine's anti-inflammatory activity in the *in vivo* models of arthritis.[137, 138] However, the full anti-inflammatory potential of narciclasine and the molecular mechanisms responsible for this action have not been fully revealed so far.

In the present work narciclasine is investigated as a small molecule which might interfere with the interaction between leukocytes and endothelial cells. This is an important step in the development of inflammation. In all experiments endothelial cells have been treated with narciclasine. Two steps of this interaction, leukocyte adhesion and transmigration through the endothelial cell layer, were studied *in vitro* in more detail. The influence of narciclasine on the expression of the cell adhesion molecules (ICAM-1, VCAM-1, E-selectin) and fractalkine (CX3CL1), which are responsible for facilitation of leukocyte's adhesion, was measured. *In vivo* leukocytes rolling, adhesion and transmigration were investigated *via* intravital microscopy. The murine model for acute peritonitis was used as an *in vivo* model for acute inflammation and pain.

This study investigated molecular mechanisms responsible for narciclasine's anti-inflammatory effects. In particular, all steps of the NF-κB signaling pathway were examined. The influence of narciclasine on TNF-triggered NF-κB activation cascade and the expression of the TNF receptor 1 were investigated.

Taken together, narciclasine's property to interfere with the leukocyte-endothelial cell interaction was investigated together with the underlying molecular mechanisms responsible for its anti-inflammatory action.

2 MATERIALS & METHODS

2.1 Materials

2.1.1 Narciclasine

Narciclasine was purchased from Carl Roth (Karlsruhe, Germany). The compound was obtained from the plant *Narcissus pseudonarcissus*. Narciclasine stock solution was prepared by dissolving narciclasine in dimethyl sulfoxide (DMSO). This solution was stored in a freezer at -20 °C.

For experiments, narciclasine was diluted in the cell culture medium. The concentration of DMSO did not exceed 0.1 % (v/v) when applied to the mammalian cells.

2.1.2 Cell culture media

Cell culture media reagents and supplements used for experiments are listed in **Table 2**. Fetal bovine serum (FBS) was inactivated at 56 °C in a water bath before use as a component of the growth media.

Table 2: Cell culture reagents and supplements

Reagent	Manufacturer
Amphotericin B (250 µg/ml)	PAN-Biotech GmbH (Aidenbach, Germany)
Collagen G	Biochrom AG (Berlin, Germany)
Endothelial cell growth medium (ECGM)	PELOBiotech GmbH (Martinsried, Gemrany)
Fetal bovine serum	Biochrom AG (Berlin, Germany)
Leukocyte separation media	PromoCell GmbH, Heidelberg, Germany
Medium 199 (M199)	PAA Laboratories GmbH (Gölbe, Germany)
Microvascular endothelial cell growth medium (MECGM)	PELOBiotech GmbH (Martinsried, Gemrany)
Penicillin (10 000 U/ml)	PAN-Biotech GmbH (Aidenbach, Germany)
RPMI-1640 medium	PELOBiotech GmbH (Martinsried, Gemrany)
Streptomycin (10 mg/ml)	PAN-Biotech GmbH (Aidenbach, Germany)
Trypsin/EDTA (T/E)	Biochrom AG (Berlin, Germany)

Cell culture media used in the *in vitro* experiments with its complete composition can be found in **Table 3**.

Table 3: Cell culture media used in experiments

Culture media	Components	Composition
Endothelial cell growth media (ECGM) complete media	FBS Penicillin Streptomycin Amphotericin B ECGM	10 % 100 U/ml 100 µg/ml 2.5 µg/ml
Freezing Medium	DMSO FBS	10 %
Microvascular EC media	FBS Penicillin Streptomycin MECGM	10 % 100 U/ml 100 µg/ml
RPMI-1640 complete	FBS Penicillin Streptomycin RPMI-1640	10 % 100 U/ml 100 µg/ml
Stopping media	FCS M199	10 %

2.1.3   Cells used in the *in vitro* experiments

Primary cells and cell lines used in *in vitro* experiments are listed in **Table 4**.

Table 4: Primary cells and cell lines

Name	Cell Type	Manufacturer
HUVEC	Primary human umbilical vein endothelial cells	PELOBiotech (Martinsried, Germany)
hCMEC/D3	Human cerebral microvascular endothelial cells	Merck Millipore (Darmstadt, Germany)
THP-1	Monocyte-like cell line	Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany)
Jurkat	Lymphocyte-like cell line	Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany)

2.1.4 Animals for *in vivo* experiments

Table 5 contains information about the animals used in intravital microscopy and zymosan-induced model of acute peritonitis. In both experiments, mice were used as animal models.

Table 5: Animals used in *in vivo* experiments

Species	Sex	Strain	Source
Mice	female	C57BL/6N	Charles River (Sulzfeld, Germany)
Mice	male	C57BL/6J	Charles River (Sulzfeld, Germany)

2.1.5 Biochemicals, reagents, inhibitors and dyes

Biochemicals, reagents, inhibitors and dyes used for the experiments are listed in **Table 6**.

Table 6: Biochemicals, reagents, inhibitors and dyes

Reagent	Manufacturer
Ammonium persulfate (APS)	Carl Roth GmbH (Karlsruhe, Germany)
ε-Aminocaproic acid	Sigma-Aldrich (Taufkirchen, Germany)
Bovine serum albumin (BSA)	Sigma-Aldrich (Taufkirchen, Germany)
Calcium chloride dihydrate $CaCl_2$ x 2 H_2O	Carl Roth GmbH (Karlsruhe, Germany)
Citrate	Carl Roth GmbH (Karlsruhe, Germany)
cOmplete™, Mini, EDTA-free	Roche Diagnostics (Mannheim, Germany)
p-coumaric acid	Sigma-Aldrich (Taufkirchen, Germany)
CellTracker™ Green CMFDA dye	Life Technologies GmbH (Darmstadt, Germany)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (Taufkirchen, Germany)
Disodium hydrogen phosphate dihydrate Na_2HPO_4 x 2 H_2O	Carl Roth GmbH (Karlsruhe, Germany)
Dithiothreitol (DTT)	Sigma-Aldrich (Taufkirchen, Germany)
o-Dianisidine	Sigma-Aldrich (Taufkirchen, Germany)
dNTP-set, peqGOLD	VWR International GmbH (Darmstadt, Germany)
Ethanol	Sigma-Aldrich (Taufkirchen, Germany)
Formaldehyde, 16% ultrapure	Polyscience Europe GmbH (Eppelheim, Germany)
FluorSave™ Reagent	Merck Millipore (Darmstadt, Germany)
Glycine	Carl Roth GmbH (Karlsruhe, Germany)
β-glycerophosphate disodium salt hydrate	Sigma-Aldrich (Taufkirchen, Germany)
HOECHST 33342	Sigma-Aldrich (Taufkirchen, Germany)
Hexadecyltrimethylammoniumbromide	Sigma-Aldrich (Taufkirchen, Germany)

Table 6: Biochemicals, reagents, inhibitors and dyes (continued)

Reagent	Manufacturer
Hydrogen peroxide solution, 30% (w/w) H_2O_2	Sigma-Aldrich (Taufkirchen, Germany)
Interleukine 1β (IL-1 β)	PeproTech GmbH (Hamburg, Germany)
Kolliphor HS 15	Sigma-Aldrich (Taufkirchen, Germany)
Luminol	Sigma-Aldrich (Taufkirchen, Germany)
Magnesium chloride hexahydrate $MgCl_2$ x 6 H_2O	Merck Millipore (Darmstadt, Germany)
Methanol	VWR International GmbH (Darmstadt, Germany)
β-Mercaptoethanol	Carl Roth GmbH (Karlsruhe, Germany)
Narciclasine	Sigma-Aldrich (Taufkirchen, Germany)
Nonidet P-40	Sigma-Aldrich (Taufkirchen, Germany)
Penicillin/Streptomycin mix	PAN-Biotech GmbH (Aidenbach, Germany)
Potassium chloride KCl	Merck Millipore (Darmstadt, Germany)
Potassium dihydrogen phosphate KH_2PO_4	Carl Roth GmbH (Karlsruhe, Germany)
Powdered milk	Carl Roth GmbH (Karlsruhe, Germany)
Power SYBR® Green PCR Master Mix	Applied Biosystems (Wattham, MA, USA)
Phenylmethylsulfonyl fluoride (PMSF)	Roche Diagnostics (Mannheim, Germany)
Propidium Iodide (PI)	Sigma-Aldrich (Taufkirchen, Germany)
Protein-Marker IV ('Prestained'), 10 – 170 kDa, peqGOLD	VWR International GmbH (Darmstadt, Germany)
Pyronin Y	Sigma-Aldrich (Taufkirchen, Germany)
Random Primer 6	New England BioLabs GmbH (Frankfurt am Main, Germany)
Recombinant RNasin® RNase Inhibitor	Promega Corporation (Mannheim, Germany)
Roti®-Histofix, 4%	Carl Roth GmbH (Karlsruhe, Germany)
Rotiphorese® Gel 30	Carl Roth GmbH (Karlsruhe, Germany)
RNase AWAY®	Molecular BioProducts Inc. (San Diego, CA, USA)
Sodium chloride (NaCl)	Carl Roth GmbH (Karlsruhe, Germany)
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH (Karlsruhe, Germany)
Sodium fluoride (NaF)	Sigma-Aldrich (Taufkirchen, Germany)
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich (Taufkirchen, Germany)
Sodium pyrophosphate decahydrate $Na_4P_2O_7$ x 10 H_2O	Sigma-Aldrich (Taufkirchen, Germany)
Staurosporine	Sigma-Aldrich (Taufkirchen, Germany)
SuperScript™ II Reverse Transcriptase	Thermo Fischer Scientific (Dreieich, Germany)
SYBR Green PCR Master Mix	Life Technologies (Darmstadt, Germany)
Tetramethylethylenediamine (TEMED)	Bio-Rad Laboratories (Hercules, CA, USA)
Tris	Carl Roth GmbH (Karlsruhe, Germany)
TNF-α (human recombinant)	PeproTech GmbH (Hamburg, Germany)
Triton® X-100	Merck Millipore (Darmstadt, Germany)

Table 6: Biochemicals, reagents, inhibitors and dyes (continued)

Reagent	Manufacturer
Tween® 20	Sigma-Aldrich (Taufkirchen, Germany)
Zymosan A	Sigma-Aldrich (Taufkirchen, Germany)

2.1.6   Buffers and solutions

Buffers and solutions that were used for western blot, qRT-PCR, immunofluorescent staining and *in vivo* experiments are listed in **Table 7**.

Table 7: Buffers and solutions

Buffer	Complete composition	
Anode buffer I (pH 10.4)	Tris	300 nM
	Methanol	20 % (v/v)
	dH_2O	
Anode buffer II (pH 10.4)	Tris	25 nM
	Methanol	20 % (v/v)
	dH_2O	
Blocking buffer	BSA	0.2 %
	PBS (pH7.4)	
Blotto-T (1 % / 5 %)	Powdered milk	1 % / 5 %
	TBS (pH 7.4)	
5 % BSA	BSA	5 %
	TBS (pH 7.4)	
Cathode buffer	Tris	25 nM
	ε-Aminocaproic acid	40 mM
	Methanol	20 % (v/v)
	dH_2O	
CellTracker™ Green solution	CellTracker™ Green CMFDA dye	5 µM
	RPMI-1640	
DNase I mix	DNase I (RNase-free DNase Set)	12.5 %
	RDD (RNeasy® Mini kit)	87.5 %
Electrophoresis Buffer	Glycine	192 mM
	Tris	25 mM
	SDS	0.1 %
	dH_2O	
70% Ethanol	Ethanol	70 %
	RNase-free water	30 %
HBSS (pH 7.4)	NaCl	136.9 mM
	KCl	5.4 mM
	$NaHCO_3$	4.2 mM
	D-Glucose	0.5 mM
	KH_2PO_4	0.4 mM
	$Na_2HPO_4 \times 2\,H_2O$	0.3 mM
	dH_2O	

Table 7: Buffers and solutions (continued)

Buffer	Complete composition	
HFS solution	Sodium citrate	0.1 %
	Triton® X-100	0.1 %
	Propidium iodide	74.8 nM
	PBS (pH 7.4)	
Homemade ECL	Luminol	1 % (v/v)
	p-coumaric acid	0.4 %
	Tris (pH 8.5)	100 mM
	H_2O_2	0.02 % (v/v)
	dH_2O	
HTAB in PB	HTAB	0.5 %
	PB (pH 6.0)	
Laemmli sample buffer, 1 x / 5 x	Tris-HCl (pH 6.8)	125 / 312.5 mM
	Glycerol	20% / 50% (w/v)
	SDS	4% / 5% (w/v)
	DTT	0.8% / 2% (w/v)
	Pyronin-Y	0.01% / 0.025%
	dH_2O	
Modified RIPA lysis buffer	cOmplete®	4 mM
	NaF	1 mM
	Na_3VO_4	0,3 mM
	PMSF	1 mM
	Sodium phosphate decahydrate	10 mM
	β-glycerophosphate disodium salt hydrate	3 mM
	H_2O_2	0.12 %
	RIPA (pH 7.6)	
MPO substrate solution	o-Dianisidine	0.3 mM
	H_2O_2	0.0009 %
	PB (pH 6.0)	
PB (pH 6.0)	KH_2PO_4	67 mM
	$Na_2HPO_4 \times 2\ H_2O$	84 mM
	dH_2O	
PBS (pH 7.4)	NaCl	137 mM
	KH_2PO_4	2 mM
	KCl	2.7 mM
	$Na_2HPO_4 \times 2\ H_2O$	10 mM
	dH_2O	
PBS+ (pH 7.4)	NaCl	136.9 mM
	KH_2PO_4	10.6 mM
	KCl	2.6 mM
	$Na_2HPO_4 \times 2\ H_2O$	1.3 mM
	$CaCl_2 \times 2\ H_2O$	0.7 mM
	$MgCl_2 \times 6\ H_2O$	0.5 mM
	dH_2O	
PBSA	BSA	0.2 %
	PBS (pH 7.4)	
PBS/Collagen G	Collagen G	0.25 %
	PBS (pH 7.4)	

Table 7: Buffers and solutions (continued)

Buffer	Complete composition	
RIPA (pH 7.6)	NaCl	150 mM
	Tris-HCl (pH 7.5)	50 mM
	Nonidet P40	1 %
	Sodium deoxycholate	0.25 %
	SDS (10%)	0.1 %
	dH$_2$O	
RIPA lysis buffer	cOmplete®	4 mM
	NaF	1 mM
	Na$_3$VO$_4$	1 mM
	PMSF	1 mM
	RIPA (pH 7.6)	
RLT lysis buffer	β-mercaptoethanol	1 %
	RLT (RNeasy® Mini kit)	
Separation gel, 10%	Rotiphorese® Gel 30	33.3 %
	Tris-HCl (pH 8.8)	150 mM
	SDS	0.1 %
	APS	0.05 %
	TEMED	0.1 %
	dH$_2$O	
Stacking gel, 4%	Rotiphorese® Gel 30	17 %
	Tris-HCl (pH 8.8)	125 mM
	SDS	0.1 %
	APS	0.1 %
	TEMED	0.2 %
	dH$_2$O	
Stripping buffer	Tris-HCl (pH 8.8)	62.5 mM
	SDS	2 %
	β-mercaptoethanol	0.8 %
	dH$_2$O	
Tank buffer	Glycine	192 mM
	Tris	25 mM
	Methanol	10 %
	dH$_2$O	
TBS (pH 7.4)	Tris	25 mM
	NaCl	150 mM
	dH$_2$O	
TBS-T	Tween® 20	0.05 %
	TBS (pH 7.4)	
Zymosan A suspension	Zymosan A	2 mg/ml
	PBS	

2.1.7   Oligonucleotides

Information about the oligonucleotides used in qRT-PCR experiments is provided in **Table 8**.

Table 8: Oligonucleotides used for qRT-PCR

Gene Name	Gene Product	Primers
ACKR-1	Atypical Chemokine Receptor 1	Forward 5'-GTG CCA TGG GGA ACT GTC TG-3' Reverse 5'-TTC CAG GTT GGC ACC ATA GTC-3'
COX2	Cyclooxygenase-2	Forward 5'-TGG GGT GAT GAG CAG TTG TT -3' Reverse 5'-GGA TGC CAG TGA TAG AGG GTG -3'
CX3CL1	Fractalkine	Forward 5'-CAC CAC GGT GTG ACG AAA TG-3' Reverse 5'-TTG ACC CAT TGC TCC TTC GG-3'
DUSP-1	Dual specificity phosphatase 1	Forward 5'-TGC AGT ACC CCA CTC TAC GA-3' Reverse 5'-GGT TGT CCT CCA CAG GGA TG-3'
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase	Forward 5'-CCA CAT CGC TCA GAC ACC AT-3' Reverse 5'-TGA AGG GGT CAT TGA TGG CAA-3'
ICAM-1	Intracellular cell adhesion molecule 1	Forward 5'-CTG CTC GGG GCT CTG TTC-3' Reverse 5'-AAC AAC TTG GCC TGG TCA CA-3')
Gene Name	Gene Product	Primers
SELE	E-selectin	Forward 5'-AGA TGA GGA CTG CGT GGA GA-3' Reverse 5'-GTG GCC ACT GCA GGA TGT AT-3'
VCAM-1	Vascular cell adhesion molecule 1	Forward 5'-CCA CAG TAA GGC AGG CTG TAA-3' Reverse 5'-GCT GGA ACA GGT CAT GGT CA-3

2.1.8   Antibodies

Antibodies used in western blot experiments are listed in **Table 9**.

Table 9: Antibodies used in western blot experiments

Target antigen	Manufacturer	Catalog #	Working concentration
β-actin	Sigma-Aldrich (Taufkirchen, Germany)	A3854	1:100000
Anti-goat	R&D Systems (Minneapolis, Minnesota, USA)	HAF017	1:1000
Anti-rabbit	Life Technologies (Carlsbad, CA, USA)	A11008	1:400
Anti-mouse	Cell Signaling Technology/New England Biolabs (Frankfurt, Germany)	7076	1:2000
Anti-rabbit	Cell Signaling Technology/New England Biolabs (Frankfurt, Germany)	7074	1:5000
COX2	Santa Cruz Biotechnology (Dallas, Texas, USA)	sc-1745	1:200
CX3CL1	eBioscience – Thermo Fisher Scientific (Darmstadt, Germany)	14-7986-81	1:1500

Table 9: Antibodies used in western blot experiments (continued)

DUSP-1	Santa Cruz Biotechnology (Dallas, Texas, USA)	Sc-370	1:200
E-selectin	Santa Cruz Biotechnology (Dallas, Texas, USA)	sc-137054	1:500
ICAM-1	Santa Cruz Biotechnology (Dallas, Texas, USA)	sc-107	1:500
IL1R-1	Santa Cruz Biotechnology (Dallas, Texas, USA)	sc-13160	1:500
IKKβ	Cell Signaling Technology/New England Biolabs (Frankfurt, Germany)	8943	1:1000
IκBα	Cell Signaling Technology/New England Biolabs (Frankfurt, Germany)	9242	1:1000
JNK	Cell Signaling Technology/New England Biolabs (Frankfurt, Germany)	9258	1:1000
Phospho-IKKα/β	Cell Signaling Technology/New England Biolabs (Frankfurt, Germany)	2697	1:1000
Phospho-IκBα	Cell Signaling Technology/New England Biolabs (Frankfurt, Germany)	2859	1:1000
Phospho-JNK	Cell Signaling Technology/New England Biolabs (Frankfurt, Germany)	9255	1:2000
Phospho-p38	Cell Signaling Technology/New England Biolabs (Frankfurt, Germany)	4511	1:1000
phospho-TAK-1	Cell Signaling Technology/New England Biolabs (Frankfurt, Germany)	4508	1:1000
p38	Cell Signaling Technology/New England Biolabs (Frankfurt, Germany)	9212	1:1000
TAK-1	Cell Signaling Technology/New England Biolabs (Frankfurt, Germany)	4505	1:1000
TNFR1	Cell Signaling Technology/New England Biolabs (Frankfurt, Germany)	3736	1:1000
VCAM-1	Cell Signaling Technology/New England Biolabs (Frankfurt, Germany)	sc-13160	1:300

Information about the antibodies used for flow cytometric analysis is provided in **Table 10**.

Table 10: Antibodies used for flow cytometric analysis

Target antigen	Manufacturer	Catalog #	Working concentration
CD11b	BD Bioscience (Heidelberg, Germany)	564985	1 µg
E-selectin	BD Bioscience (Heidelberg, Germany)	551145	1:10
FCγ-receptor (CD16/32)	BD Bioscience (Heidelberg, Germany)	553142	1:50
I-A/I-E	BD Bioscience (Heidelberg, Germany)	562564	1 µg
ICAM-1	Bio-Rad Laboratories GmbH, Munich, Germany	MCA1615F	1:33

Table 10: Antibodies used for flow cytometric analysis (continued)

Target antigen	Manufacturer	Catalog #	Working concentration
Ly-6C	BD Bioscience (Heidelberg, Germany)	560592	1 µg
Ly-6G	BD Bioscience (Heidelberg, Germany)	551460	1 µg
VCAM-1	BD Bioscience (Heidelberg, Germany)	555647	1:20

For immunofluorescent staining antibodies from **Table 11** were used.

Table 11: Antibodies used in immunohistochemistry

Target antigen	Manufacturer	Catalog #	Working concentration
NF-κB p65	Santa Cruz Biotechnology (Dallas, Texas, USA)	sc-327	1:400
Alexa Fluor® 488	Thermo Fisher Scientific (Dreieich, Germany)	A-11008	1:400

2.1.9 Plasmids

Plasmids used in the Dual-Luciferase Reporter Gene Assay are listed in **Table 12**.

Table 12: Plasmids

Vector	Manufacturer	Catalog #
pGL4.32[*luc2P*/NF-κB/RE/Hygro]	Promega GmbH (Mannheim, Germany)	E8491
pGL4.74[*hRluc*/TK]	Promega GmbH (Mannheim, Germany)	E6921

2.1.10 Commercial kits

Commercial kits are listed in **Table 13**.

Table 13: Commercial Kits

Kit Name	Manufacturer
CytoTox 96® Non-Radioactive Cytotoxicity Assay	Promega GmbH (Mannheim, Germany)
Dual-Luciferase® Reporter Assay System	Promega GmbH (Mannheim, Germany)
Mouse/Rat Soluble Protein Master Buffer Kit RUO	BD Bioscience (Heidelberg, Germany)
Mouse TNF Flex Set RUO	BD Bioscience (Heidelberg, Germany)

Table 13: Commercial Kits (continued)

Mouse IL-6 Flex Set RUO	BD Bioscience (Heidelberg, Germany)
Mouse IL-1β Flex Set RUO	BD Bioscience (Heidelberg, Germany)
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific GmbH, Dreieich, Germany
RNase-Free DNase Set	Qiagen GmbH (Hilden, Germany)
RNeasy® Mini Kit	Qiagen GmbH (Hilden, Germany)

2.1.11 Technical equipment

Technical equipment that was used during this work is listed in **Table 14**.

Table 14: Technical Equipment

Device	Description	Manufacturer
ARPEGE 110	Liquid nitrogen storage	Air Liquide S.A. (Paris, France)
Astacus	Ultra pure water system	MembraPure GmbH (Berlin, Germany)
CP 1000	Tabletop film processor	AGFA (Cologne, Germany)
Criterion® Blotter	Blotting unit	Bio-Rad Laboratories (Hercules, CA, USA)
DM IL LED	Inverted microscope	Leica (Wetzlar, Germany)
DMI 6000 B	Inverted fluorescence microscope	Leica (Wetzlar, Germany)
Dynamic Weight Bearing 2.0	Automated dynamic weight bearing device	Bioseb (Boulogne, France)
FACSVerseTM	Flow cytometer	BD Bioscience (Heidelberg, Germany)
FE20	pH-measurement device	Mettler Toledo (Columbus, USA)
FORMA 900	Freezer (-80 °C)	Thermo Fisher Scientific (Dreieich, Germany)
HERACell 150i	Incubator	Thermo Fisher Scientific (Dreieich, Germany)
HERAEUS MEGAFUGE 16R	Centrifuge	Thermo Fisher Scientific (Dreieich, Germany)
IKA® RH basic 2	Magnetic stirrer	IKA-Werke GmbH & Co. KG (Staufen, Germany)
Infinite F200 Pro	Microplate multifunction reader	Tecan (Männendorf, Switzerland)
Ibidi Pump System	Pump System	Ibidi (Martinsried. Germany)
Julabo ED	Water bath	JULABO GmbH (Seelbach, Germany)
MICRO STAR 17R	Microcentrifuge	VWR International GmbH (Darmstadt, Germany)
Mini-PROTEAN® Short Plates	Glass plate	Bio-Rad Laboratories, Inc. (Hercules, CA, USA)
Mini-PROTEAN® Spacer Plates	Glass spacer plate	Bio-Rad Laboratories, Inc. (Hercules, CA, USA)

Table 14: Technical Equipment (continued)

Device	Description	Manufacturer
Mini-PROTEAN Tetra Cell	Gel electrophoresis cell	Bio-Rad Laboratories, Inc. (Hercules, CA, USA)
NanoPhotometer® P330	Spectrophotometer	Implen GmbH (Munich, Germany)
Nucleofactor 2b	Electroporation device	Lonza (Basel, Switzerland)
Olympus BX 50 upright microscope	Microscope	Olympus Microscopy (Hamburg, Germany)
PowerPacᴛᴍ HC	Power supply	Bio-Rad Laboratories (Hercules, CA, USA)
SAFE 2020	Clean bench	Thermo Fischer Scientific (Dreieich, Germany)
Sonopuls HD 70	Sonicator	BANDELIN electronic GmbH & Co. KG (Berlin, Germany)
StepOnePlusTM Real-Time PCR System	Real-time PCR system	Applied Biosystems (Waltham, MA, USA)
Stuartᴛᴍ SRT9D	Tube roller mixer	Bibby Scientific Limited (Staffordshire, UK)
Systec X-95	Autoclave	Systec GmbH (Linden, Germany)
Trans-Blot® TURBO™ transfer system	Blotting chamber	Bio-Rad Laboratories, Inc. (Hercules, CA, USA)
USC300TH	Ultrasonic water bath	VWR International GmbH (Darmstadt, Germany)
Varioskan Flash	Microplate multifunction reader	Thermo Fisher Scientific (Dreieich, Germany)
Vortex Genie 2	Vortex mixer	Scientific Industries Inc. (Bohemia, US-NY)
VWR® Digital Dry Block Heaters	Heat block	VWR International GmbH (Darmstadt, GER)

2.2 Cell culture

All cells were cultivated in the presence of 5 % CO_2 at 37 °C. The composition of the cell culture media and freezing media is listed in **Table 3**. The components of phosphate buffer saline (PBS) and PBS/Collagen G (PBS/collagen G) are listed in **Table 7**. Different "n" in the experiments with cell lines stands for the number of different passages of the same cells line. For primary cells, "n" represents number of different donors of the cells.

ECs were cultivated in ECGM. During passaging or the actual experiments cell culture flasks, well-plates and inserts were coated with PBS/Collagen G for at least 20 min at 37 °C prior to seeding of the cells.

Primary leukocytes as well as THP-1 and Jurkat cell lines were cultivated in the RPMI-1640 complete media.

2.2.1   Human umbilical vein endothelial cells

Primary human umbilical vein endothelial cells (HUVECs) were used as a model for endothelial cells. HUVECs were purchased form PELOBiotech (Martinsried, Germany). For the cultivation of HUVECs, ECGM was supplemented with heat-inactivated fetal calf serum (FCS), penicillin, streptomycin and amphotericin B **(Table 3)**. HUVECs used for the experiments were in passage three.

2.2.2   Human cerebral microvascular endothelial cells

Human cerebral microvascular endothelial cells (hCMEC/D3) were used as a model of ECs that form the blood-brain barrier in humans.[143] The cell line was purchased from Merck Millipore (Darmstadt, Germany). The primary isolate derived from the human temporal lobe microvessels of an epilepsy patient, was enriched in the cerebral endothelial cells.[144] hCMEC/D3 were cultured in microvascular EC growth medium **(Table 3)**. They were used for experiments between passages three and eight. hCMEC/D3 were used for the adhesion assay and detection of the surface protein expression of intracellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1).

2.2.3   Human leukemia monocyte-like cell line THP-1

THP-1 cells were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were isolated from the peripheral blood of a one-year old male patient with acute monocytic leukemia.[145] THP-1 were cultivated in the RPMI-1640 complete **(Table 3)**. Cells were used in the experiments up to passage 30. THP-1 cells were used in adhesion and transmigration assays.

2.2.4   Human leukemia lymphocyte-like cell line Jurkat

Jurkat cells were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). These cells were isolated from the peripheral blood of a male patient suffering from acute T cell leukemia.[146] They were cultured in the RPMI-1640 complete **(Table 3)**. Jurkat cells were used up to passage 30 in adhesion assay.

2.2.5   Peripheral blood mononuclear cells and polymorphonuclear lymphocytes

Human peripheral blood mononuclear cells (PBMCs) and polymorphonuclear lymphocytes (PMNLs) were isolated from buffy coats purchased from Deutsches Rotes Kreuz, Blutspendedienst Baden-Württemberg/Hessen, Institut für Transfusionsmedizin und Immunhämatologie (Frankfurt, Germany). The procedure was performed according to a modified method developed by Boyum.[147] The method is based on the ability of plasma cells to distribute gradually after centrifugation. The content of buffy coat was mixed with PBS 1:1 and transferred into the tube containing 10 ml 5 % dextran. After 30 min erythrocytes sedimented to the bottom of the tube and the upper phase containing PMBCs and PMNLs was transferred to the new tube on top of the leukocyte separation medium. Then tube was centrifuged for 10 min without break at 800g, RT.

After centrifugation, PBMCs were visible as a white ring and PMNLs sedimented to the bottom of the tube. To isolate PBMCs, the white ring was transferred into a new tube and washed twice with ice-cold Hank's balanced salt solution. PBMCs were then washed three times with ice-cold RPMI-1640. Freshly isolated PBMCs were used for cell adhesion assays directly on the day of the isolation.

To isolate PMNLs, their sediment was first resuspended in 1 ml ice cold PBS. Then 50 ml of ice-cold PBS was added to the tube containing PMNLs and the tube was centrifuged at 300 g for 10 min with break at RT. After the supernatant was aspirated with the vacuum pump, 10 ml of ice-cold Milli Q water was added to the cell pallet and the ballet was vortexed for 45 seconds to lyse erythrocytes, which might be left in the cell mixture. Then ice-cold PBS (40 ml) was added into the

tube. Afterwards cells were centrifuged at 200 g for 10 min (without break) at RT. The lysis of erythrocytes was repeated one more time. After the last centrifugation step, cell pallet was resuspended in RPMI-1640 complete. PMNLs were stored on ice until they were used in the cell adhesion assays on the same day. Isolation of PMNLs was performed by Melissa Krishnathas from the Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany

2.2.6   Primary lymphocytes

To obtain primary lymphocytes, PBMCs in RPMI-1640 were seeded onto 6-well plates (Sarstedt AG & Co. KG, Nümbrecht, Germany). After 1.5 h monocytes adhered to the bottom of the plate. Primary lymphocytes stayed in the suspension with RPMI-1640. The cell suspension was collected from each well. Cells were centrifuged at 300 g for 5 min at RT and resuspended in complete RPMI-1640. After 24 h, primary lymphocytes were used for cell adhesion assays.

2.2.7   Passaging of the cells

ECs were sub-cultured 1:3 in 75 cm_2 culture flasks or seeded onto cell culture well-plates, slides or inserts for experiments. To start the sub-culturing, cells were first washed twice with PBS. Then T/E (trypsin/EDTA) solution was applied for 2 min to facilitate the detachment of the cells form the surface of the cell culture flask. This enzymatic reaction was stopped by adding stopping medium. After the centrifugation (300 g, 5 min, RT), ECs were resuspended in the cell culture medium. HUVEC and hCMEC/D3 were split 1:3 twice a week.

The suspension cell lines (THP-1 and Jurkat cells) were passaged by adjusting the end concentration in the 75 cm_2 culture flasks to $3 \times 10_5$ and $5 \times 10_5$ cells/ml respectively. Cells were centrifuged at 300 g for 5 min at RT. Afterwards, they were resuspended in complete RPMI-1640 and cultivated until further passaging or were used for experiments. The passaging was performed three times a week.

2.2.8   Freezing and thawing of the cells

For freezing, adherent cells were first detached from the surface of the cell culture flask as described in the **section 2.2.7** of ***Materials and Methods***. Suspension cells were removed from the culture medium by centrifugation (300 g, 5 min, RT). Then, cells were resuspended in 2 ml of ice-cold freezing medium, transferred into cryovials and kept at -80 °C overnight. On the next day cryovials were transferred to liquid nitrogen for longer-term storage.

To thaw the cells, cryovials were put into a warm water bath (37 °C). Then, they were transferred dropwise into a tube, containing 9 ml of RT-warm cell culture media. After centrifugation (300 g, 5 min, RT), cells were resuspended in warm culture medium and cultured in a 75 cm$_2$ cell culture flask (adherent cells) or a 25 cm$_2$ cell culture flask (suspension cells).

2.3   Cytotoxicity assays

To identify the safe dose range in which narciclasine could be applied to endothelial cells, the cytotoxicity of narciclasine was tested in HUVECs. Narciclasine was tested in the concentration range between 0.01 and 3 µM.

2.3.1   Apoptosis

Apoptotic cells were detected according to the method developed by Nicoletti *et al.*[148] The method is based on the quantification of the number of cells containing sub-diploid DNA after staining with propidium iodide (PI), which binds to the DNA. To perform this assay, confluent HUVECs were treated as indicated for 24 and 48 h. Then, the supernatants were collected and cells left in the plate were washed twice with PBS. After each washing PBS solution was not discarded but was added to the respective cell culture supernatant. Remaining cells were detached from the well using Trypsin/EDTA solution applied for 2 min at 37 °C. Enzymatic reaction was stopped by adding stopping medium. Detached cells were centrifuged together with supernatants which were collected earlier (600 g, 10 min, 4 °C). Cells from the pellet were washed twice with PBS. All obtained cells were incubated overnight at 4 °C in HFS solution, which contained PI. The percentage of the

apoptotic cells was measured using a FACS Verse (BD) flow cytometer and analyzed by the software BD FACSuit.

2.3.2   LDH release

The CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Heidelberg, Germany) was used to identify if narciclasine had cytotoxic effects on endothelial cells. This assay is based on the measurement of the lactate dehydrogenase (LDH) release as this is a hallmark of cytotoxic cell death.[149] LDH is a cytosolic enzyme, which gets released from the cell after loss of its cell membrane integrity. During the assay LDH, released form cells, converts the added tetrazolium salt (iodonitrotetrazolium violet) into a red formazan product. The number of lysed cells is proportional to the amount of formazan that is formed during the reaction. For this assay, confluent HUVECs were treated with different concentrations of narciclasine for 24 and 48 h. LDH release was measured following the manufacturer's protocol. A maximum release of LDH was used as positive control, which was provided in the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit. Absorbance was measured on a Varioskan Flash plate reader (Thermo Fischer Scientific) at 490 nm.

2.4   Cell adhesion assay

To test the influence of narciclasine on the adhesion of immune cells to the surface of endothelial cells, cell adhesion assays were performed under flow and static conditions.

Adhesion assay under flow conditions. Cell adhesion assay under flow conditions was performed by Dr. Rebecca Ingelfinger and Dr. Iris Bischoff-Kont (Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany). HUVECs (1.6×10^6 cells / ml) were cultivated in µ-slides $I_{0.8}$ Luer (Ibidi, Martinsried, Germany) under flow conditions (5 dyn/cm^2). Cells were cultured under continuous shear stress conditions for 24 h using an Ibidi Pump System. Endothelial cells were pretreated with narciclasine (300 nM). After 30 min they were activated with TNF (10 ng/ml) for 24 h. THP-1 (8×10^5 cells / ml) cells were fluorescently labeled with

Cell Tracker Green and were added to the flow system. They were allowed to adhere to HUVEC for 10 min under a flow rate of 2 ml/min. THP-1 that did not adhere were removed with PBS+. After the last washing step, PBS+ was left on the surface and phase contrast images were obtained using a DM IL Light Emitting Diode inverted microscope. Once PBS+ was removed, HUVEC with adherent THP-1 cells were lysed. After this, fluorescence was measured with a Tecan Infinite F200 Pro microplate reader (ex: 485 nm, em: 535 nm).

Adhesion assay under static conditions. HUVECs or hCMEC/D3 were seeded on the surface of a 24 well-plate. After 48 h, confluent ECs were pre-treated with narciclasine (10, 30, 100, 300 nM) for 30 min and then activated with TNF (10 ng/ml) for 24 h. Where in indicated, HUVECs were treated with narciclasine 6 h after TNF. Adhesion assay with HUVEC and THP-1 or Jurkat cells were performed by Dr. Rebecca Ingelfinger and Dr. Iris Bischoff-Kont (Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany).

THP-1 cells, Jurkat cells, PBMCs or primary lymphocytes were washed twice with RPMI-1640 (w/o FCS) and stained with Cell Tracker Green for 30 min at 37 °C in the same medium. After staining cells were washed two times in PRMI-1640 (w/o FCS). Leukocytes were resuspended in ECGM containing 10 % heat-inactivated FCS, 100 U/ml penicillin, 100 U/ml streptomycin and 0.25 µg/ml amphotericin B and seeded onto the endothelial cell layer. Adherence time for the different cell types is listed in Error! Reference source not found.. After the end of the corresponding incubation time, ECs were washed twice with PBS+. In case of THP-1 cells, Jurkat cells, PBMCs and PLs the fluorescence from adherent cells was measured with a Tecan Infinite F200 Pro plate reader (ex: 485 nm, em: 535 nm) directly after washing steps.

Table 15: Adhesion time of the leukocytes to endothelial cells.

Leukocyte type	Endothelial cell type	Adhesion time
THP-1 cells	HUVEC	1 h, 5 min*
	hCMEC/D3	10 min
Jurkat cells	HUVEC	1 h, 5 min*
	hCMEC/D3	10 min
PBMCs	HUVEC	7 min
PL	HUVEC	5 min
PMNL	HUVEC	30 min

* - represents experimental cases when HUVECs were treated with narciclasine after activation by TNF. In all other cases narciclasine was added 30 min before TNF.

Adhesion of PMNLs (1 x 10^4 cells / well) to HUVECs was also determined under static conditions. The amount of adherent PMNLs was determined using myeloperoxidase activity measurement. Adhesion assays with HUVECs and PMNLs were performed by Melissa Krishnathas from the Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany. After adhesion for 30 min, PMNLs that did not adhere were washed twice with PBS+. Afterwards, cells in the well were lysed in 110 µl 0.5 % HTAB in PB. After incubation for 30 min at 37 °C lysates were frozen at -80 °C until measurement. On the day of the measurement, lysates were thawed and 50 µl of the lysates per well were transferred into a 96-well plate. 50 µL of MPO substrate solution was added to each well. Enzyme activity was determined immediately by the absorption measurement using an Infinite F200 pro microplate reader (Tecan) at 450 nm. The measurement was performed every 30 s over 30 min. The linear part of the absorption curve was used to perform a linear regression analysis. The amount of adherent PMNLs was calculated using the slope obtained from the linear regression.

2.5 Transmigration assay

Transmigration assays were performed to investigate the influence of narciclasine on the transmigration of leukocytes through the endothelial cell layer *in vitro*. This assay was performed by Dr. Rebecca Ingelfinger (Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany).

HUVECs were seeded onto the upper compartment of the Transwell® Inserts (pore size 8 µm, diameter 6.5 mm, polycarbonate membrane) and cultured for 24 h. Then HUVECs were treated as indicated with narciclasine and activated with TNF (1 ng/ml) for 24 h. For each well 1 x 10^5 of HUVECs in 100 µl ECGM (10 % heat-inactivated FCS, 100 U/ml penicillin and 100 U/ml streptomycin) were used. Prior to the transmigration, THP-1 (2 x 10^4 per well) were stained for 30 min with Cell Tracker Green (5µM). In the upper compartment of all inserts, medium 199

supplemented with 0.1 % BSA was used. In the lower compartment, medium 199 supplemented with 500 ng/ml in total of SDF-1 (CXCL12) was applied to generate a chemotactic gradient. THP-1 cells were allowed to transmigrate through the HUVECs monolayer for 2 h. Then, the upper compartment of the insert was cleaned from the cells that did not transmigrate. Inserts were put into 200 µl of RIPA lysis buffer and frozen at -80 °C for at least 1 h. After plates with inserts were thawed, 60 µl of the cell lysates were taken for fluorescence measurements with a Tecan Infinite F200 Pro plate reader (ex: 485 nm, em: 535 nm).

2.6 Cell surface protein expression measured by flow cytometry

Flow cytometry was performed to identify the levels of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin surface protein expression of HUVECs and intracellular cell adhesion molecule-1 (ICAM-1) and VCAM-1 surface protein expression of hCMEC/D3. HUVEC or hCMEC/D3 were grown until confluence for 48 h in a 12-well plate. Then, ECs were pre-treated with different concentrations of narciclasine as indicated. After 30 min, ECs were treated with TNF (10 ng/ml) for 4 h (E-selectin) or 24 h (ICAM-1, VCAM-1).

To prepare samples for the detection of VCAM-1 and E-selectin surface protein expression, HUVECs and hCMEC/D3 were washed twice with PBS and incubated with 200 µl/well Accutase (commercially available solution of proteolytic enzymes) at 37 °C for 3 min. Afterwards, 800 µl of Medium 199 (10 % FCS, 100 U/ml penicillin, 100 U/ml streptomycin) were added to each well. The obtained cell suspension was centrifuged (300 g, 5 min, 4 °C). After washing of the cells with ice-cold PBS, they were centrifuged (300 g, 5 min, 4 °C). The next washing step was performed with PBSA. Then, cells were incubated with the respective antibody (anti-VCAM-1, FITC-labeled; anti-E-selectin, PE-labeled) in PBSA for 45 min at 4 °C in dark. Cells were washed twice with PBS. Surface protein expression was measured with a FACSVerse (BD) flow cytometer.

In case of the detection of ICAM-1 surface protein expression, hCMEC/D3 were washed twice with PBS and treated with 200 µl of trypsin/EDTA per well at 37 °C

for 3 min. Then cells were transferred to flow cytometry tubes containing 200 µl of 4 % formaldehyde in PBS and incubated for 10 min at RT. After this, cells were centrifuged for 5 min at 1200 rpm at RT. Cells were washed with 1 ml PBS and centrifuged again under the same conditions. Cells were stained with FITC-labeled anti-ICAM-1 antibody for 45 min at 4 °C in dark. After the end of the incubation time, cells were washed with PBS, and surface protein expression was measured with a FACSVerse (BD) flow cytometer.

2.7 Quantitative reverse transcription polymerase chain reaction

The principle of the quantitative reverse transcription polymerase chain reaction (qRT-PCR) is based on the measurement of the fluorescent signal from the fluorescent dye, in this research SYBR Green, from an amplified product of the gene of interest. This measurement is possible because of the ability of SYBR Green to emit fluorescent light after its binding to double-stranded DNA (dsDNA).[150] Before performing the qPCR, mRNA was isolated and then it was transcribed into the complementary DNA (cDNA) using reverse transcriptase. This allowed the direct correlation between the obtained signal from the SYBR Green bound to the obtained dsDNA product to the mRNA expression of the gene.

In this research, qRT-PCR was used to identify the influence of narciclasine on the gene expression of ICAM-1, VCAM-1, E-selectin, CX3CL1, atypical chemokine reseptor-1 (ACKR1-1), dual-specificity phosphatase-1 (DUSP-1) and cyclooxygenase 2 (COX2). All experiments were performed with HUVECs. Commercial kits, which were used for mRNA isolation, cDNA synthesis and qPCR performance are listed in **Table 13**.

2.7.1 Preparation of the samples for qPCR

HUVECs were seeded onto 6-well plates and grown until confluence for 24 h. Afterwards, the cell culture medium was aspirated, and cells were washed twice with PBS. Then 350 µl of RLT lysis buffer were given onto the HUVECs. Plates were frozen at -80 °C until further usage.

2.7.2   Isolation of the messenger RNA

Prior to the start of RNA isolation, all surfaces were cleaned with RNase AWAY®
to avoid the degradation of the RNA by ribonucleases and to remove RNA that
does not come from the analyzed samples. All tubes used during the isolation were
RNase-free. The isolation of the mRNA was performed according to the protocol
provided in the RNAeasy® Mini Kit. Briefly, cell lysates were thawed on ice and
transferred into 1.5 ml tubes. Then, 70% ethanol was added in the proportion 1:1
to the sample amount. The content of the tubes was carefully mixed by rotation
and transferred into tubes provided by the kit. These tubes contained the RNA-
binding membrane. After centrifugation for 1 min at 13.300 g the supernatant was
discarded, and the membrane was washed with 350 µl RW1 washing buffer. To
avoid DNA contamination of the samples, the DNase digestion step was
performed. 80 µl of DNase I mix was added onto the membrane of the column.
After incubation of the samples for 15 min at RT, the columns were washed with
350 µl of RW1 washing buffer and centrifuged for 1 min at 13.300 g. The
supernatants were discarded, and the columns were washed with 500 µl of RPE
buffer. After centrifugation (13.300 g, 1 min), samples were washed again with
500 µl of RPE and centrifuged again (13.300 g, 2 min). Columns were put into
DNase and RNase free tubes. 30 µl of double distilled DNase and RNase free
water was added on the surface of the columns and they were centrifuged (13.300
g, 1 min). RNA concentration was determined using a NanoPhotometer® P330.
Isolated mRNA was either frozen at -80 °C or used immediately for the synthesis
of the complementary DNA (cDNA).

2.7.3   Synthesis of the complementary DNA

For cDNA synthesis, 1 µg RNA from each sample was used. Samples were filled
up to 7 µl with DNase- and RNase-free water. 1 µl of Random Primer 6 and 4 µl of
deoxyribonucleotides (dNTPs, 2 mM) were added to each sample. Samples were
incubated for 10 min at 70 °C. Afterwards, they were put for 2 min on ice. Each
sample was incubated for 10 min at RT with 8 µl of Master Mix containing 1 µl of
RNasin, 4 µl of 5 x First Strand Buffer, 2 µl of 100 nM DTT and 1 µl SuperScript II

Reverse Transcriptase (Thermo Fischer Scientific). Afterwards, samples were incubated for 1 h at 42 °C. 80 µl of DNase- and RNase-free water were added to each sample and they were incubated for another 10 min at 70 °C. Samples were either frozen at -20 °C or used directly for quantitative PCR.

2.7.4 Performance of qPCR

cDNA samples were diluted 1:5. Total volume after the dilution was 2.5 µl in each well. Primers for qPCR were diluted 1:25 prior to use. 1.25 µl of every primer (forward and reverse) was applied per sample. The list of primers used in the experiments provided in **Table 8**. 5 µl of SYBR® Green Mix were used per sample. Each sample was pipetted on the MicroAmpTM Optical 96-well plate in technical triplicates. qPCR was performed using equipment from the StepOnePlusTM System (Applied Biosystems). The program used in the thermocycler can be found in the **Table 16.** Glyceraldehyde-3-phosphate (GAPDH) was used as housekeeping gene for the normalization of the mRNA expression. Acquired data was quantified using the comparative $2^{-\Delta\Delta CT}$ method.[151]

Table 16: Thermocycler program performance for qPCR

Step of the qRT-PCR	Temperature	Time	Number of Cycles
Activation of the DNA-polymerase	95 °C	10 min	1
DNA denaturation	95 °C	15 s	40
Primers annealing/elongation	60 °C	60 s	40
Melt curve state	95 °C	15 s	
	60 °C	60 s	1
	95 °C	15 s	
Hold	4 °C		1

2.8 Western blot analysis

Western blot analysis was used for protein detection. This method was first developed by Towbin *et al.*[152] During the procedure proteins from cell lysates are transferred to a membrane after they have been separated by sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins of interest are detected by the corresponding antibody.

In this research, western blot analysis was used to identify the influence of narciclasine on the protein expression of ICAM-1, VCAM-1, E-selectin, CX3CL1, COX2, DUSP-1, phospho-JNK, phospho-p38, phospho-TAK-1, TAK-1, phospho-IKKβ, IKKβ, phospho-IκBα, IκBα, TNF receptor 1 and IL-1 receptor 1. Experiments were performed with on HUVECs. Buffers and equipment used for all steps of western blot analysis are listed in **Table 7** and **Table 14** respectively.

2.8.1   Preparation of samples for western blot analysis

HUVECs were seeded into 6-well plates and grown until confluence. Then they were treated with narciclasine as indicated and activated with TNF (10 ng/ml). Detailed description of the narciclasine concentrations and treatment times are provided in **Table 17.** After incubation with TNF, the cells were washed once with PBS and lysed with RIPA lysis buffer. For the detection of phosphorylated forms of proteins, modified RIPA lysis buffer was applied. Plates were frozen at -80 °C for 1 h or overnight.

Table 17: HUVEC treatment schemes for western blot analysis

Protein	Narciclasine concentration (nM)	Pre-incubation with narciclasine	Activation with TNF
ICAM-1	10, 30, 100, 300	30 min	24 h
VCAM-1	10, 30, 100, 300	30 min	24 h
E-selectin	10, 30, 100, 300	30 min	4 h
CX3CL1	10, 30, 100, 300	30 min	24 h
COX2	100, 300	30 min	24 h
DUSP-1	30, 100, 300	2 h	8 h
JNK	300	2 h	5 min, 30 min, 2 h
p38	300	2 h	5 min, 30 min, 2 h
IκBα	300	6 h 5 min, 6h, 8 h, 24 h	5 min, 30 min, 2 h -
IKKβ	300	6 h 5 min, 6h, 8 h, 24 h	5 min, 30 min, 2 h -
TAK-1	300	6 h 5 min, 6h, 8 h, 24 h	5 min, 30 min, 2 h -
TNFR1	300	6 h 5 min, 6h, 8 h, 24 h	5 min, 30 min, 2 h -
IL-1R1	300	6 h	-

2.8.2   Protein quantification

Protein quantification was performed according to the protocol from Pierce™ BCA Protein Assay Kit. The method is based on the ability of proteins to reduce Cu_{2+} to Cu_+ in an alkaline environment.[153] One cuprous ion Cu_+ chelates two molecules of bicinchoninic acid (BCA), which results in the formation of a purple colored reaction product. The protein concentration is linear proportional to the amount of the formed BCA/copper complex.

To identify the concentration of the isolated protein content, cell lysates were first transferred from the wells into 1.5 ml tubes. The tubes were incubated for 5 min in the ultrasonic water bath USC300TH or, in case of the nuclear protein analysis, sonicated for 10 s with a Sonopuls HD 70 sonicator. Then, the protocol from the Pierce™ BCA Protein Assay Kit was followed. Samples were diluted with 1x Laemmli sample buffer so that the same concentration of protein could be applied during the gel electrophoresis.

2.8.3   Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) allows to separate proteins according to their molecular weight based on their electrophoretic mobility.[154] Thus, proteins with lower molecular weight move faster through the gel compared to proteins with a higher weight. Location of the protein on the membrane in correspondence to the location of the proteins of marker proteins with known molecular weights is a defining feature for protein detection.

SDS-PAGE was performed using a vertical electrophoresis system. For all samples, 10 % acrylamide gels were used. Samples moved through the stacking gel at 80 V for 30 min. When the samples reached the separation gel, the voltage was increased until 120 V for 1.5 h (for detection of ICAM-1, VCAM-1, E-selectin, COX2, CX3CL1, DUSP-1, JNK, p38) or until 200 V for 50 min (for detection of IKKβ, IκBα, TAK-1, TNFR1, IL-1R1). As a reference for the molecular weight of the proteins, the peqGOLD Protein Marker IV (10-170 kDa) was used.

2.8.4 Immunoblotting

The compositions of the buffers used for tank or semi-dry blotting are listed in **Table 7**. The order of the layers in the sandwich, blotting conditions and proteins, which were detected by tank or semi-dry blotting, can be seen in the **Table 18**.

Table 18: Immunoblotting methods used in this research

	Tank blotting	Semi-dry blotting
Sandwich composition	• cathode-fiber pad • filter paper soaked in tank buffer • separating gel • PVDF membrane • soaked in tank buffer • fiber pad-anode	• filter paper soaked in anode buffer 1 • filter paper soaked in anode buffer 2 • separating gel • PVDF membrane • filter paper soaked in cathode buffer
Blotting conditions	100V, 1h, 4 °C	30 min, RT
Detected proteins	ICAM-1, VCAM-1, E-selectin, CX3CL1, COX2, DUSP-1, JNK, p38	(p)IκBα, (p)IKKβ, (p)TAK-1, TNFR1, IL-1R1

Protein transfer from the gel to the polyvinylidene difluoride (PVDF) membrane was performed either by tank blotting or by semi-dry blotting using in both cases equipment from Bio-Rad laboratories GmbH. Prior to protein transfer, PVDF membranes were activated in methanol for 5 min.

2.8.5 Protein detection

The membranes were blocked for 2 h at RT on a roller in either 5 % Blotto or 5 % BSA to block unspecific binding sites. Primary antibodies were applied on the membranes overnight at 4 °C. After detection of the proteins of interest, an anti-β-actin antibody was applied for 1 h at RT as a control for the assay. Antibodies used in this research for western blot analysis are listed in **Table 9**. The membranes were washed five times with the washing buffer and then incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h. Unbound secondary antibody was removed by washing the membrane five times with the

washing buffer. After this, the membranes were incubated for 1 min with Homemade ECL solution. The combination of the luminol, H_2O_2 in the solution and HRP bound to the membrane, results in luminescence, which can be detected after exposure of the membrane to an X-ray film. Development of the films was performed by an X-ray film processor CR 1000. Images obtained after the scanning of the X-ray films were quantified with the software Image J (version 1.49, National Institutes of Health, Bethesda, MD, USA).

2.8.6   Membrane stripping

Stripping off the antibody from the membrane for further application of another antibody was applied in cases when the molecular weight of the proteins detected on the same membrane was similar. For stripping, the membrane was incubated in the stripping solution containing freshly added β-mercaptoethanol for 30 min at 50 °C. Then, the membrane it was blocked by either 5 % Blotto-T or 5 % BSA. The procedure continued as described before in the part **2.8.5**.

2.9   Immunocytochemistry

Immunofluorescent staining was used to identify whether narciclasine influenced the nuclear translocation of the NF-κB p65 subunit. HUVEC were seeded onto 8-well µ-slides (ibidi, Martinsried, Germany) and grown to confluence for 24 h. They were pre-treated with 300 nM of narciclasine for 0.5, 1, 2, 6, 12, 24 h. Experiments where HUVECs were pre-treated with narciclasine for 1 and 2 h were performed by Dr. Rebecca Ingelfinger from the Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany. Then, cells were activated with TNF (10 ng/ml) for 30 min. Each well was washed three times with PBS and fixed with Roti-Histofix® for 10 min and permeabilized with 0.2 % Triton X-100 in PBS for 2 min. Then, cells were incubated in PBS containing 0.2% BSA for 20 min to block unspecific binding sites. After three washing steps, anti-NF-κB p65 antibody (1:400) in PBS containing 0.2% BSA was applied to cells. Cells were incubated with the anti-NF-κB p65 antibody on a shaker for 2 h at RT in the dark. Afterwards, cells were incubated with AlexaFluor® 488-linked secondary antibody (1:400) on a shaker for 1 h at RT in dark. After three washing steps, cells were embedded into

FluorSaveₜₘ mounting medium. µ-Slides were examined by a fluorescence microscope (Leica DMI6000 B). ImageJ software version 1.49k was used for quantification of fluorescence signals.

2.10 Dual-luciferase reporter gene assay

Reporter Gene Assays are used for the detection of gene expression, activity of receptors, transcription factors, intracellular signaling, mRNA processing and protein folding.[155, 156] A special kind of Reporter Gene Assay "Dual-Luciferase Reporter Assay" allows to detect the simultaneous expression of two reporter enzymes. One of these enzymes is located in a vector that contains response elements for transcription factor of interest. The second reporter enzyme is in another vector, which encodes luciferase reporter gene *hRluc*. The activity of the second reporter enzyme serves as an internal control. At the end of the experiment, experimental reporter activity is normalized to the activity of the internal control. This helps to minimize the differences which are caused by different transfection efficiency and cell viability. In our experiments, Dual-Luciferase Reporter Assay was used to investigate the influence of narciclasine on the NF-κB promoter activity.

2.10.1 Transfection of the cells

Transfection protocol was adapted from Lonza (Basel, Switzerland). Briefly, 1 Mio. HUVECs were co-transfected with 4.32 µg of a vector coding for the NF-κB response elements (pGL4.32[luc2P/NFκB-RE/Hygro]) and *Photinus* luciferase (Promega) and 1.93 µg of a vector coding for *Renilla* luciferase (Promega) (pGL4.74[hRluc/TK]). To transfect the cells electroporation was performed. For this Nucleofector 2b Device from Lonza was used. After transfection, 560 µl of ECGM were added into each cuvette and this volume was distributed to 8 wells in a 48-well plate. After 5 h, cells were washed carefully with PBS+, and 65 µl of 1 x PLB was added to each well. Plates were frozen for 1 h or overnight at -80 ∘C.

2.10.2 Luminescence measurements

Cells were thawed on a shaker, and the whole content of a well was transferred into a 1.5 ml tube and centrifuged for 3 min at 13.000 rpm at RT. 10 µl from the supernatant were transferred into white flat bottom 96-well plate for measurement. LAR II and Stop&Glow solution from Dual-Luciferase Reporter Assay System (Promega) were prepared according to manufacturer's protocol. For each well 50 µl of LAR II and 50 µl of Stop&Glow solutions were used. Luminescence measurement was performed on Tecan Infinite F200 Pro.

2.11 Zymosan-induced *in vivo* model of acute peritonitis

Work on this model was performed in collaboration with Prof. Dr. Dr. Achim Schmidtko and Gesine Wack from the Institute of Pharmacology and Clinical Pharmacy, Goethe University, Frankfurt/Main, Germany. Gesine Wack performed *in vivo* experiments and data analysis for dynamic weight bearing. Prof. Dr. Dr. Achim Schmidtko provided scientific supervision.

Zymosan-induced inflammation is an established model for acute peritonitis in mice.[157, 158] Acute peritonitis results in the increased infiltration of monocytes and neutrophils to the peritoneal cavity of mice. Inflammation created in the abdomen causes pain. To detect pain caused by the inflammation, dynamic weight bearing measurements are applied.[159]

2.11.1 Animals

Acute peritonitis model experiments were performed with 8-12 weeks old female C57BL/6N mice. Mice were purchased from Charles River (Sulzfeld, Germany). Animals were housed with free access to water and food on a 12/12-hour light/dark cycle. All experiments were approved by the local Institutional Animal Care and Use Committee (Regierungspräsidium Darmstadt) under the reference number V54-19c20/15-FR/1004 and adhered to the guidelines of the International Association for the Study of Pain.

2.11.2 Zymosan-induced peritonitis

Mice were taken from the animal house to the experimental room 24 h before the start of the experiment. First, narciclasine (1 mg/kg) or vehicle (10% Kolliphor and 1 % DMSO in 0.9 % NaCl) were subcutaneously injected in the neck of mice 12 h prior to the zymosan injection. The volume used per mouse was 100 µl. Acute peritonitis in mice was induced by a single intraperitoneal (i.p.) injection (0.5 ml) of a zymosan A suspension (2 mg/ml) in PBS+ as described.[157]

2.11.3 Dynamic weight bearing measurement

The dynamic weight bearing (DWB) assessment was performed in naive mice (baseline) and 5 h after the zymosan injection. Spontaneous pain caused by zymosan-induced peritonitis was detected by an automated DWB device (Bioseb, Boulogne, France). The device represents an acrylic glass cage (11 cm wide x 11 cm long x 20 cm high). The floor of the cage was supplied with weight sensors, which allow to measure the weight borne in each limb. For an additional control, there was a camera installed above the cage. The camera helped to locate the exact position of the animal during the recording to make sure that the sensor in the floor really measured the pressure produced by the mouse paw. For the analysis of the recorded weight distribution the DWB software was used as described.[159, 160] The following parameters were selected to consider a zone a valid weight distribution zone: ≥ 0.8 g on one captor with minimum of two adjacent captors recording ≥ 1.0 g. A time segment of ≥ 3 stable pictures was considered as valid. The validation of the positions of the paws on the video sequences were done manually by an observer, blinded for the treatment of the animals. To quantify the experiments, the front-paw to hind-paw ratio was calculated using the mean of the weight over the time. The front-paw to hind-paw ratio at baseline, which exceeded 0.5, was considered an exclusion criterion.

2.11.4 Obtaining of peritoneal lavage from mice

Peritoneal lavage was collected from the ketamine/xylazine anaesthetized mice six hours after the zymosan injection. During the procedure the outer skin of the

peritoneum was gently pulled back, allowing the access to the inner skin lining of the peritoneal cavity. After the injection of 2 ml of ice-cold sterile PBS with 27 g needle, the abdomen of mice was gently massaged. After 2 min, the peritoneal lavage fluid was removed with a 25 G needle, transferred to the 1.5 ml tubes and kept on ice. Mice were killed during anesthesia by cervical dislocation. The lavage was centrifuged at 13.000 rpm, 4 °C for 5 min. The supernatants were collected for cytokine measurements, and cells were used for the analysis of cell populations.

2.11.5 Measurement of the monocytes and neutrophils in the peritoneal lavage

Peritoneal lavage was analyzed for presence of monocytes and neutrophils, which migrated into the abdominal cavity as reaction to the inflammation caused by zymosan. All antibodies and the Fcγ-receptor blocking cocktail for the lavage analysis were purchased from BD Bioscience, Heidelberg, Germany (for more details see **Table 10**).

Firstly 4 x 10^5 cells from each animal, separated from the supernatant, were washed with ice-cold PBS. Before incubation with the antibodies, cells were incubated with the Fcγ-receptor (CD16/32) blocking cocktail in PBS containing 0.2 % bovine serum albumin (PBSA) for 5 min to prevent binding of the antibodies to FcγIII/II receptors, which are expressed on natural killer cells, monocytes, macrophages and dendritic cells. After 5 min, the following antibodies were added to each tube: anti-I-A/I-E (anti-MHCII, BV 421), anti-CD11b (APC), anti-Ly-6C (PE), anti-Ly-6G (FITC). Cells were incubated with the antibodies for 30 min in the dark. After two washing steps with PBS, the percentage of monocytes and neutrophils in the lavage was determined by flow cytometry on FACSVerse (BD Bioscience). For the analysis first a 2-parameter dot plot depicting I-A/I-E (MHC II) *vs.* CD11b was created. This was done with the purpose to separate monocytes and neutrophils from the macrophages and dendritic cells, which are known to express MHC II.[161-163] The cells positive for CD11b and negative for MHC II were selected for further analysis. The second dot plot was depicting Ly-6C *vs.* Ly-6G cell surface markers, which are specific for monocytes and neutrophils

respectively.[164, 165] The percentage of Ly-6C and Ly-6G positive cells was corresponding to the percentage of the monocytes and neutrophils in the lavage.

2.11.6 Detection of cytokines in the peritoneal lavage

Cytokine concentrations were measured in the lavage supernatants. For this, the Cytometric Bead Array (CBA) Cell Signaling Flex Set system (BD Bioscience) was used. During this assay soluble cytokines are bound to the beads *via* an antibody, which is located on the beads. Each bead has a known size and fluorescence. This fluorescence is measured after the incubation with the detection reagent and is proportional to the amount of the bound cytokine. The CBA assay allows to detect soluble cytokines at a very low concentration.[166]

The samples were prepared for analysis according to the protocol from Mouse/Rat Soluble Master Buffer Kit (**Table 13**) provided by the manufacturer. Flex Sets, containing beads labeled to capture a specific cytokine, were used (**Table 13**). The amount of the pro-inflammatory cytokines IL-1β, TNF and IL-6 was determined on a FACSVerse flow cytometer (BD Bioscience) and data were analyzed with the FCAP Array™ software version 3.0 (BD Bioscience).

2.12 Intravital microscopy

Intravital microscopy was performed by Gabriele Zuchtriegel at the Department of Otorhinolaryngology, Head and Neck Surgery and Walter Brendel Center of Experimental Medicine, University of Munich, Munich, Germany under the supervision of Dr. med. Christoph A. Reichel.

In vivo microscopy of the mouse cremaster muscle is an established model for the observation of the leukocyte interaction with the endothelial cell layer of the blood vessels. This model allows also measure the rate of leucocytes rolling, adhesion and transmigration though the endothelial cells.[167]

2.12.1 Animals

Experiments were performed with 8-10 weeks old C57BL/6J mice purchased from Charles River (Sulzfeld, Germany). Animals were housed on a 12/12-hour light/dark cycle with free access to water and food. The experiments were performed after the approval of the local Institutional Animal Care and Use Committee (Regierung von Oberbayern, reference number 55.2-1-54-2531-84-09).

2.12.2 *In vivo* microscopy

For this experiment, mice were firstly injected with narciclasine (intraperitoneally, 1 mg/kg) or drug vehicle. One hour later leukocyte recruitment to the cremaster muscle was induced by intrascrotal injection of TNF (300 ng in 400 ml of PBS). After four hours, mice were anesthetized with a ketamine/xylazine mixture prior to preparation of the cremaster muscle. The preparation process was performed as described by Baez *et al.* with minor modifications.[167] The intravital microscopy showing leukocyte rolling, firm adhesion and transmigration was performed on the Olympus BX 50 upright microscope and was subsequently quantified using Cap-Image image analysis software (Dr Zeintl, Heidelberg, Germany).

2.13 Statistical analysis

The number of independently performed experiments (n) is stated in the respective figure legend. It has different meaning in experiments with primary cells and cell lines. For primary cells (HUVEC, PBMC, PL), the number of experiments represents experiments performed with different donors of the cells. For the cell lines (hCMEC/D3, THP-1, Jurkat) the number of experiments corresponds to a different passage of the cells.

GraphPad Prism software version 5.0 (San Diego, CA, USA) was used for graph plotting and statistical analysis. One-way ANOVA, which was followed by Turkey's post-hoc test, was used for statistical comparisons. Data in bar graphs are expressed as means ± standard error of the mean (SEM). In the DWB analysis

statistical evaluation was done with SPSS (IBM SPSS, Chicago, IL, USA) for Windows. The normal distribution of data within groups was determined using the Kolmogorov-Smirnov test. Student's t test was used for normally distributed data analysis. These data are shown as mean ± SEM. Statistical significance was assumed at P ≤ 0.05.

3 RESULTS

3.1 Anti-inflammatory activity of narciclasine in *in vivo* experiments

3.1.1 Narciclasine reduces visceral pain, leukocyte infiltration and cytokine expression in a zymosan-induced murine peritonitis model

In vivo experiments for zymosan-induced model of acute peritonitis were performed in collaboration with Prof. Dr. Dr. Achim Schmidtko and Gesine Wack from the Institute of Pharmacology and Clinical Pharmacy, Goethe University, Frankfurt/Main, Germany. Gesine Wack performed *in vivo* experiments and data analysis for dynamic weight bearing under the scientific supervision of Prof. Dr. Dr. Achim Schmidtko.

The anti-inflammatory activity of narciclasine was tested in a murine zymosan-induced model of acute peritonitis.[157] Mice were treated with narciclasine (1 mg/kg, s.c.) for 12 h before the application of the inflammatory substance zymosan A (1 mg/kg, i.p.). After 5 h a dynamic weight bearing test was performed to assess visceral pain **(Figure 8)**. When in pain mice tend to distribute their weight more to the front paws.[159, 162] This change in the weight distribution indicates the presence of visceral pain caused by inflammation development after zymosan A injection. Mice pre-treated with narciclasine showed a significantly reduced weight distribution as compared to mice pretreated with vehicle only **(Figure 8A)**. Mice that had been treated with narciclasine put their weight more on the hind paws, which reflects their behavior under normal circumstances. Thus, it can be concluded that narciclasine reduces visceral inflammation-associated pain.

Inflammation develops when immune cells migrate to the injured area of the tissue. To identify if narciclasine had an influence on the migration of monocytes and neutrophils to the peritoneal cavity, lavage analysis was performed. The cells in the peritoneal cavity of mice were washed out with PBS and were stained with antibodies specific for each tested cell type and measured by flow cytometry. Narciclasine significantly reduced the percentage of monocytes and neutrophils in the lavage compared to vehicle-treated mice **(Figure 8B)**. Finally, the

concentration of the pro-inflammatory cytokines IL-6, TNF and IL-1β was analyzed. Narciclasine significantly reduced their presence in the analyzed lavages **(Figure 8C)**.

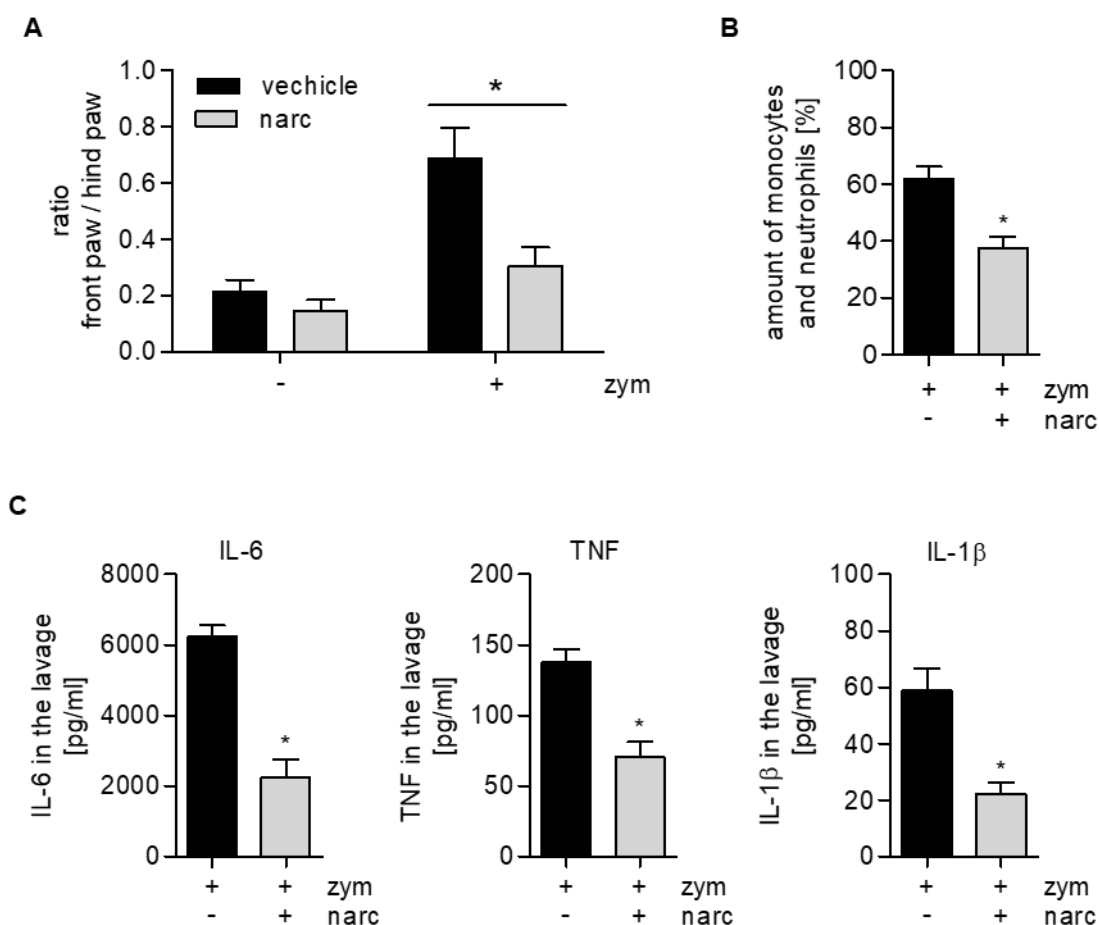These data demonstrate that narciclasine exerts anti-inflammatory properties *in vivo*.

Figure 8: Narciclasine shows anti-inflammatory effects *in vivo* in zymosan-induced murine peritonitis model.
8-12 weeks old female C57BL/6N mice were injected with narciclasine (1 mg/kg, s.c.) or vehicle (10 % Kolliphor and 1 % DMSO in 0.9 % NaCl). After 12 h, mice were treated with zymosan A (2 mg/ml, i.p.) for 5-6 h. **(A)** A dynamic weight bearing test (ratio of paw weight distribution) was performed to measure zymosan-induced abdominal pain (n=7-8). **(B)** The percentage of monocytes and neutrophils in the peritoneal lavage of mice was detected by flow cytometry (n = 4). **(C)** The concentration of the pro-inflammatory cytokines (IL-6, TNF, IL-1β) in the lavage was measured by flow cytometry using a Cytometric Bead Array system (n = 6). **(A-C)** Data are presented as mean ± SEM, *p ≤ 0.05 *vs.* vehicle **(A)** or zymosan control **(B/C)**.

3.1.2   Narciclasine reduces the TNF-triggered rolling, firm adhesion and transmigration of leukocytes *in vivo*

Intravital microscopy was performed by Gabriele Zuchtriegel at the Department of Otorhinolaryngology, Head and Neck Surgery and Walter Brendel Center of Experimental Medicine, University of Munich, Munich, Germany. Dr. med. Christoph A. Reichel supervised the experimental work. Leukocyte rolling, adhesion and subsequent transmigration through the vascular endothelium is a hallmark of inflammatory processes.[44, 45] To test if narciclasine had influence on this initial phase of inflammation, these steps of leukocyte-EC interaction were studied in the murine cremaster muscle using intravital microscopy. Mice were treated with narciclasine (1 mg/kg, s.c.) or vehicle for 1 h. Then, TNF was applied for 5 h. Intravital microscopy of the murine cremaster muscle showed that narciclasine reduces the TNF-caused leucocytes rolling, firm adhesion and their transmigration through the endothelium **(Figure 9)**. The data indicate that narciclasine interferes with the interaction between leukocytes and vascular endothelial cells after application of pro-inflammatory stimuli. Prevention of this interaction is a strong indication of the anti-inflammatory properties of narciclasine.
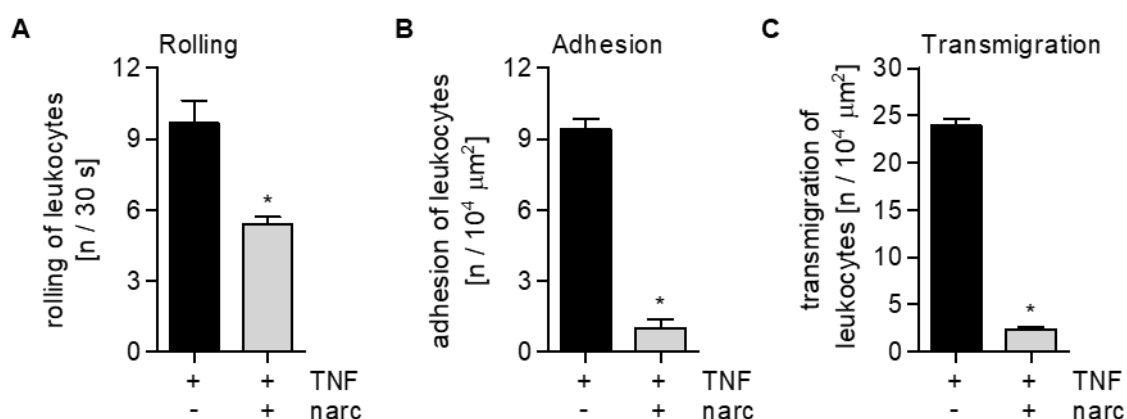
Figure 9: Narciclasine reduces the TNF-triggered rolling, adhesion and transmigration *in vivo* in the murine cremaster muscle.
C57BL/6 mice were treated with narciclasine (1 mg/kg, i.p.) or vehicle for 1 h. Then they were treated with TNF (300 ng, intrascrotal injection) for 4 h, and intravascular rolling (A), firm adhesion (B) and transmigration (C) of leukocytes on/through the inflamed tissue were analyzed in postcapillary venules of the cremaster muscle *via* intravital microscopy. Data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* TNF control.

3.2 Cytotoxicity of narciclasine

To identify the concentration of narciclasine that would be safe to use on endothelial cells *in vitro*, analysis of its cytotoxicity was performed.

3.2.1 Narciclasine does not induce apoptosis in HUEVCs in concentrations up to 1 µM

The ability of narciclasine to cause apoptotic cell death was tested using a method developed by Nicoletti.[148] The chromatin of HUVECs was stained with propidium iodide overnight. Next, the number of cells containing subdiploid DNA was measured by flow cytometry. Narciclasine did not cause apoptosis of HUVECs up to a concentration of 3 µM after 24 h and up to 1 µM after 48 h **(Figure 10)** compared to negative control. The pan-kinase inhibitor staurosporine was used as a positive control.
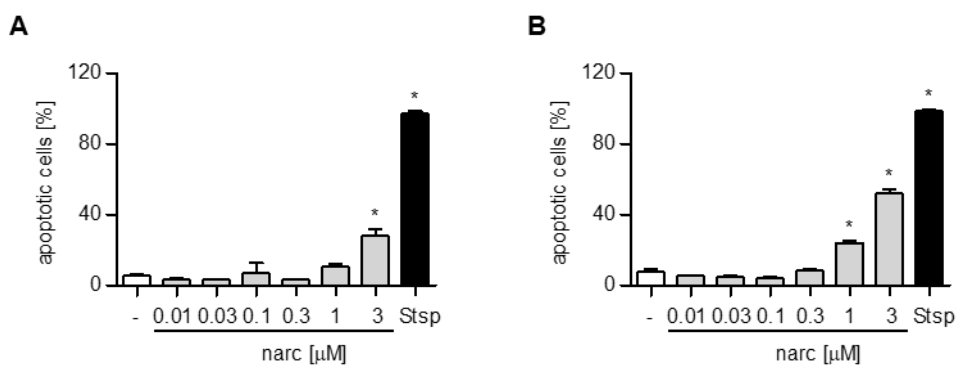
Figure 10: Narciclasine does not cause apoptosis of HUVECs up to a concentration of 1 µM.
HUVECs were treated with different concentrations of narciclasine for 24 h **(A)** and 48 h **(B)**. To measure apoptosis cells were stained with propidium iodide overnight. The number of subdiploid events (apoptotic cells) was measured by flow cytometry. Staurosporine (Stsp) (1 µg/ml) was used as positive control. CTL represents cells treated with endothelial cell growth medium only. Data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* negative control.

3.2.2 Narciclasine does not cause LDH release in HUVEC up to a concentration of 1 µM

The cytotoxicity of narciclasine was tested by measuring the release of the enzyme lactate dehydrogenase (LDH). In eukaryotic cells LDH is released by the cell as the result of the damage of its plasma membrane.[149] Narciclasine did not cause cytotoxic effects on HUVECs and the release of LDH up to a concentration of 3 µM after both 24 h and 48 h of treatment **(Figure 11)**. Considering results of narciclasine's ability to cause apoptosis and LDH release in HUVEC, it was decided to use it only in concentrations ≤ 300 nM throughout all *in vitro* experiments.
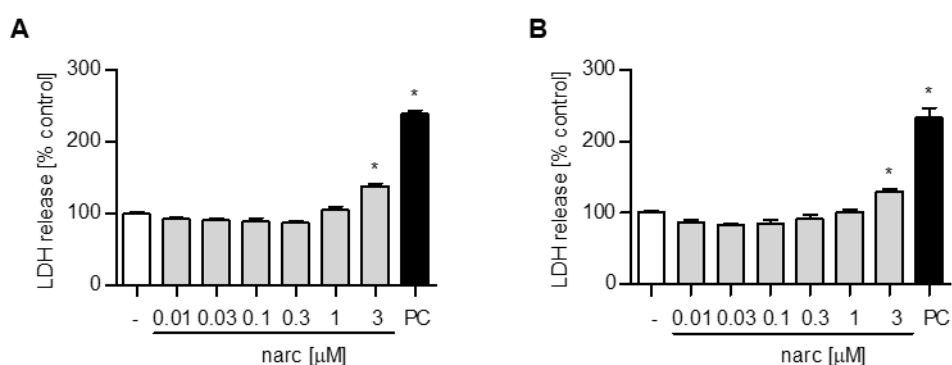
Figure 11: Narciclasine does not affect the release of LDH from HUVECs up to a concentration of 1 µM.
Confluent HUVECs were treated with different concentrations of narciclasine for 24 h **(A)** and 48 h **(B)**. To measure the level of lactate dehydrogenase (LDH) release, the Cytotox 96 non-radioactive cytotoxicity assay kit (Promega) was used. The assay was performed according to the manufacturer's instructions. Absorbance was measured at 490 nm. As positive control (PC) cells lysed with buffer provided in the kit were used. Cells treated with endothelial cell growth medium only were used as negative control (CTL). Data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* negative control.

3.3 Effects of narciclasine on the interaction between leukocytes and endothelial cells *in vitro*

The interaction between leucocytes and endothelial cells is a crucial step in the development of inflammation.[46] In this part of the research, it was demonstrated how narciclasine influences the adhesion of leukocytes and their transmigration through the EC monolayer (sections **3.3.1 – 3.3.3**).

Important molecules, that take part in these processes are ICAM-1, VCAM-1, E-selectin and CX3CL1.[48, 56] In this chapter the influence of narciclasine on all these molecules was investigated (sections **3.3.4 – 3.3.6**).

3.3.1 Narciclasine reduces the TNF-triggered adhesion of leukocytes to endothelial cells *in vitro* under flow and static conditions

Adhesion under flow conditions and data analysis were performed by Dr. Rebecca Ingelfinger and Dr. Iris Bischoff-Kont from the Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany.

Adhesion under flow conditions *in vitro* is the closest way to simulate *in vivo* adhesion where leucocytes need to attach to the endothelial cell layer, while they are floating in the blood stream. For these experiments HUVECs and monocytic-like cell line THP-1 were used. First, HUVECs were cultivated under flow conditions. Endothelial cells were pre-treated with narciclasine for 30 min and activated with TNF for 24 h. Fluorescently labeled THP-1 cells were exposed to a flow rate of 2 ml/min and were allowed to adhere for 10 min to the surface of HUVECs. From the measurement of fluorescent signal, it can be concluded that narciclasine significantly reduced THP-1 adhesion to HUVEC under flow conditions **(Figure 12)**. This finding correlates with the data obtained by intravital microscopy **(Figure 9B)**.
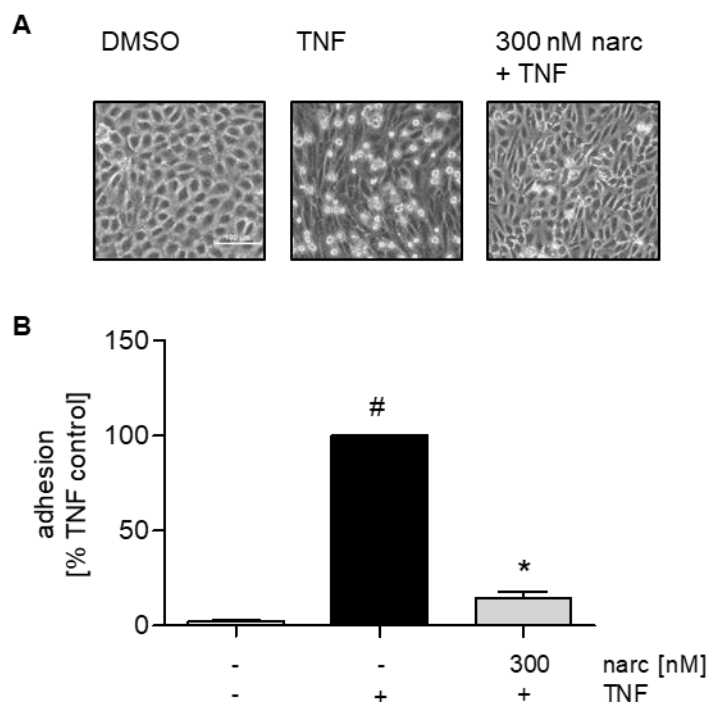
Figure 12: Narciclasine inhibits the TNF-induced adhesion of THP-1 cells to a HUVEC monolayer under flow conditions.
HUVECs, cultivated under flow conditions (5 dyn/cm2), were pre-treated with narciclasine (300 nM) for 30 min and activated with TNF (10 ng/ml) for 24 h. THP-1 cells were fluorescently labeled and were then exposed to a flow rate of 2 ml/min and allowed to adhere under these conditions for 10 min. Cells were photographed by phase contrast microscopy. The fluorescent signal from THP-1 cells was measured after cell lysis on a microplate reader. **(A)** Scale bar represents 100 μm. One representative experiment out of three is shown. **(B)** Data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* TNF control, #p ≤ 0.05 *vs.* negative control.

To investigate the effects of narciclasine on the adhesion of the leukocytes to the endothelial cells *in vitro* under the static conditions, primary ECs as well as the endothelial cell line hCMEC/D3 were used. Confluent HUVEC or hCMEC/D3 were pre-treated with narciclasine for 30 min before TNF was applied for 24 h. THP-1 cells, Jurkat cells, PBMC or primary lymphocytes were labeled with a fluorescent dye for 30 min. Afterwards they were seeded onto the endothelial cell monolayer.

The TNF-induced adhesion of PMNLs, PBMCs and PLs to HUVECs was significantly reduced by narciclasine starting from 30, 100 or 300 nM concentration respectively. **(Figure 13A)**. The TNF-triggered adhesion of Jurkat cells to hCMEC/D3 was reduced when 100 and 300 nM narciclasine was applied. The adhesion of THP-1 cells to hCMEC/D3 was inhibited at 30, 100 and 300 nM narciclasine **(Figure 13B)**.
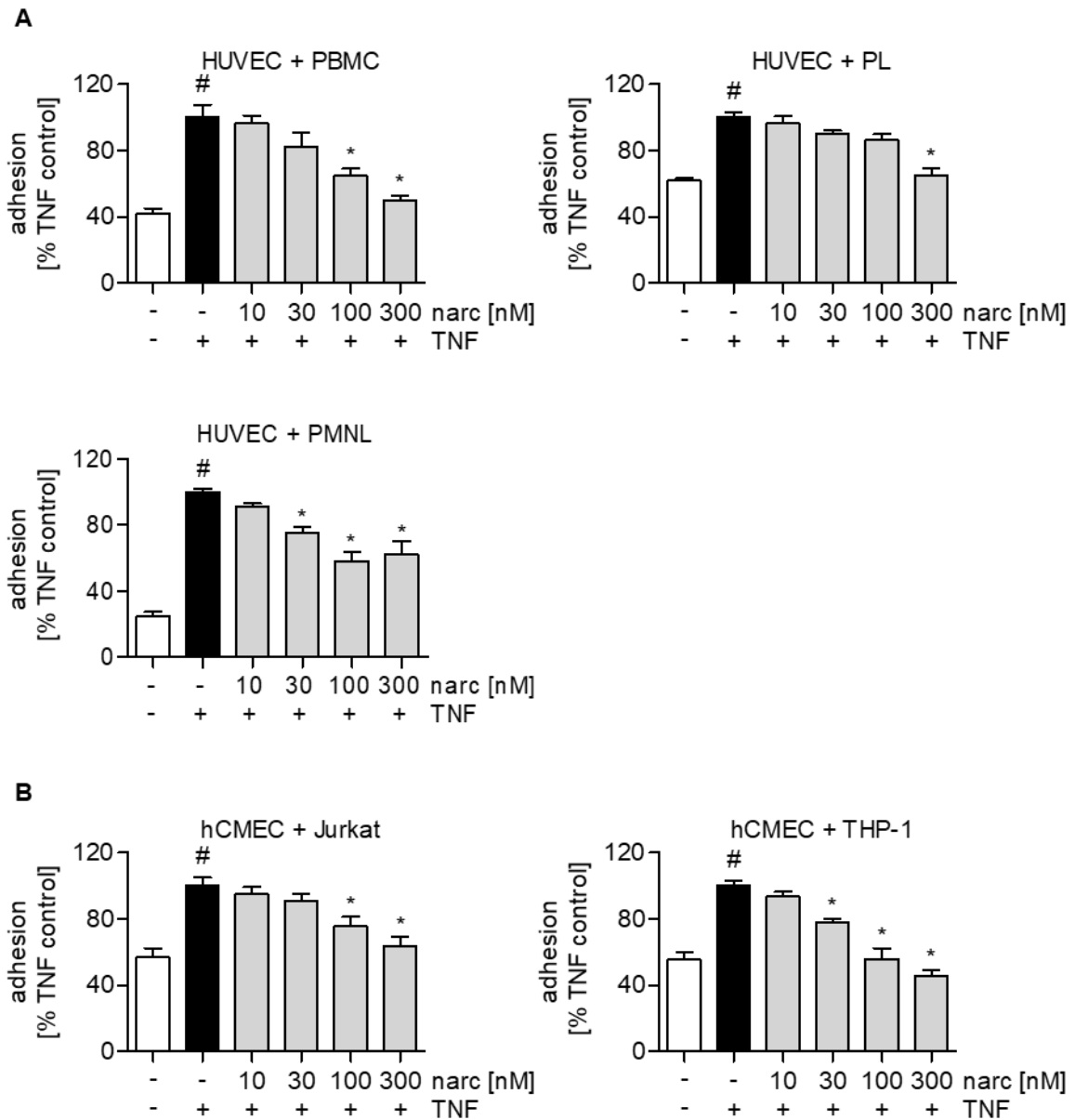
A

B

Figure 13: Narciclasine reduces the TNF-triggered adhesion of leukocytes to an endothelial cell monolayer.
Confluent HUVECs **(A)** or hCMEC/D3 **(B)** were pre-treated with narciclasine for 30 min and activated with TNF (10 ng/ml) for 24 h. THP-1 cells, Jurkat cells, PBMCs, PL or PMNLs were labeled with a fluorescent dye for 30 min. Cells were allowed to adhere to the ECs monolayer for 10 min (THP-1 cells, Jurkat cells), 7 min (PBMCs), 5 min (PL) or 30 min (PMNLs). **(A/B)** Data are expressed as mean ± SEM; n=3 (THP-1 cells, Jurkat cells, PBMCs, PLs), n=6 (PMNLs); *p ≤ 0.05 *vs.* TNF control, #p ≤ 0.05 *vs.* negative control.

Interestingly, narciclasine reduced adhesion of the THP-1 cells and Jurkat cells not only when it was added before TNF **(Figure 14A)**, but also when endothelial cells were treated with narciclasine after TNF stimulation has already started **(Figure 14B)**. Narciclasine in a concentration of 30, 100 and 300 nM significantly reduced the TNF-triggered adhesion of THP-1 cells, Jurkat cells and PBMC to HUVECs.

Data for **Figure 14** were obtained by Dr. Rebecca Ingelfinger and Dr. Iris Bischoff-Kont from the Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany.
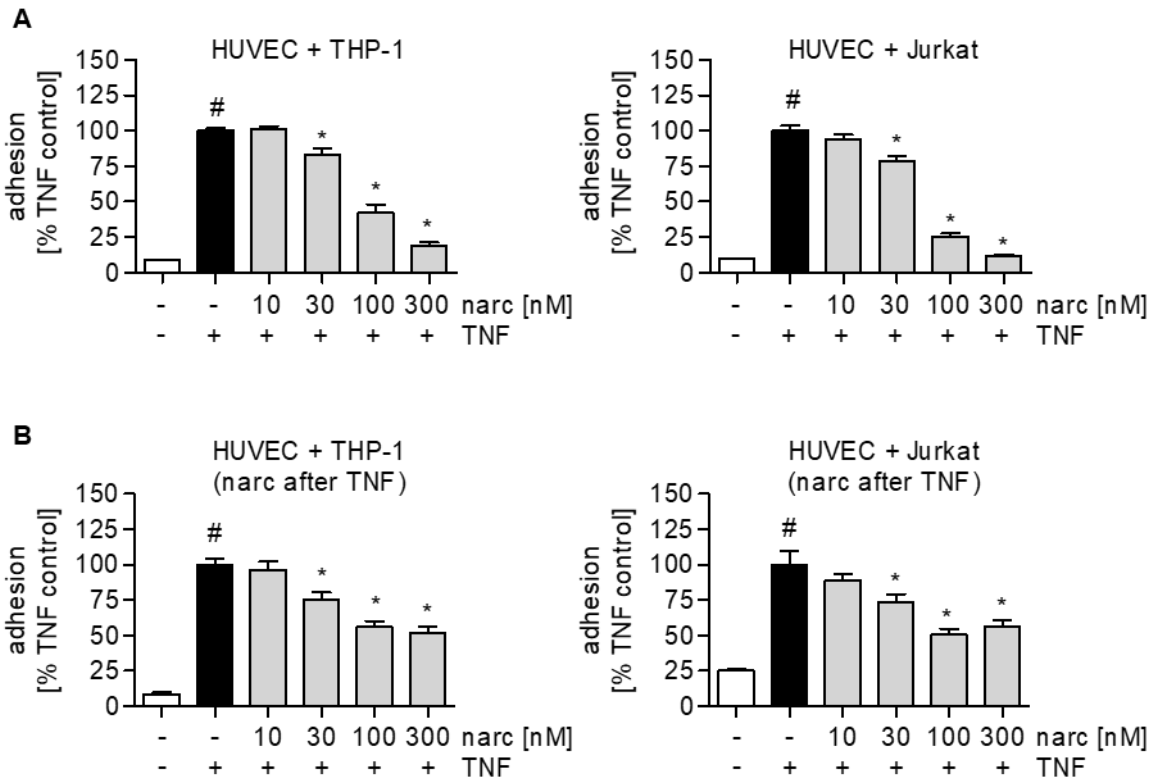

Figure 14: Narciclasine reduces adhesion of THP-1 cells and Jurkat cells when added to HUVEC monolayer before and after TNF activation.
(A) Confluent HUVECs were pretreated with narciclasine (10, 30, 100, 300 nM) for 30 min. Then they were activated with 10 ng/ml TNF for 24 h. n activated with TNF for 24 h. **(B)** HUVECs were activated with 10 ng/ml TNF. 6 h after addition of TNF narciclasine (10, 30, 100, 300 nM) was added to the culture. **(A/B)** Fluorescence-labeled THP-1 cells or Jurkat cells were added on top of the HUVECs monolayer and were allowed to adhere for 5 min. Data are expressed as mean ± SEM; n=4; *p ≤ 0.05 vs. TNF control, #p ≤ 0.05 vs. negative control.

These data demonstrate, that the ability of narciclasine to reduce adhesion of immune cells to the endothelium is independent of the type of endothelial cells and type of the immune cells or whether primary cells or cell cultures were used. Taken together all these data indicate, that narciclasine effectively reduces the TNF-triggered adhesion of leucocytes to the endothelial cell layer. This finding correlates with the results of the intravital microscopy, where adhesion of leukocytes was significantly reduced by narciclasine **(Figure 9B)**.

3.3.2 Narciclasine reduced the transmigration of leukocytes through a TNF-activated endothelial cell monolayer

Influence of narciclasine on the transmigration of THP-1 cells through a HUVECs monolayer was tested *in vitro*. This assay was performed by Dr. Rebecca Ingelfinger from the Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany. HUVECs were first seeded on the surface of the porous membrane of a Transwell insert. After 24 h, HUVECs were either treated with 300 nM narciclasine 30 min before activation with TNF **(Figure 15A)** or 6 h after **(Figure 15B).** THP-1 cells were seeded on top of the HUVEC monolayer and were allowed to transmigrate for 2 h. Prior to seeding of the THP-1 cells on the HUVEC monolayer, they were labeled with a fluorescent dye. SDF-1 was added into the lower compartment of the Transwell to create a chemotactic gradient. Narciclasine reduced the transmigration of THP-1 cells through the HUVECs monolayer **(**

Figure 15). The reduction of transmigration rate was independent of whether narciclasine was added before TNF **(**

Figure 15A) or after **(**

Figure 15B). This correlates with the results of the intravital microscopy, which demonstrated that narciclasine reduced transmigration *in vivo*. **(Figure 9C)**.
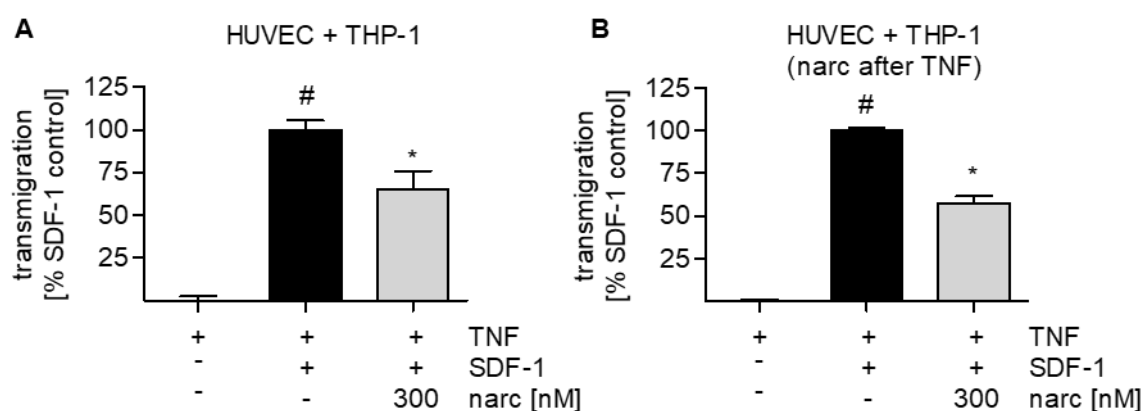

Figure 15: Narciclasine reduces transmigration of leukocytes through the endothelial cell layer.
HUVECs were grown on a porous membrane of the insert for 24 h. HUVECs were **(A)** pre-treated with narciclasine (300 nM) for 30 min before or **(B)** 6 h after the activation with TNF (1 ng/ml) for

24 h. THP-1 cells priorly labeled with the fluorescent dye, were added to the upper compartment of the insert to allow them to transmigrate through the HUVEC monolayer for 2 h. SDF-1 (500 ng/ml) was used in the lower compartment as chemoattractant for THP-1 cells. Data are expressed as mean ± SEM; **(A)** n=4; **(B)** n=5; *p ≤ 0.05 *vs.* TNF + SDF-1 control, #p ≤ 0.05 *vs.* negative control.

3.3.3 Narciclasine downregulates VCAM-1 mRNA, total protein and cell surface protein expression in TNF-activated HUVECs

The influence of narciclasine on the TNF-induced VCAM-1 mRNA and total protein expression was analyzed in HUVECs. Narciclasine starting from 30 nM significantly downregulated VCAM-1 mRNA and expression induced by TNF **(Figure 16A)**.
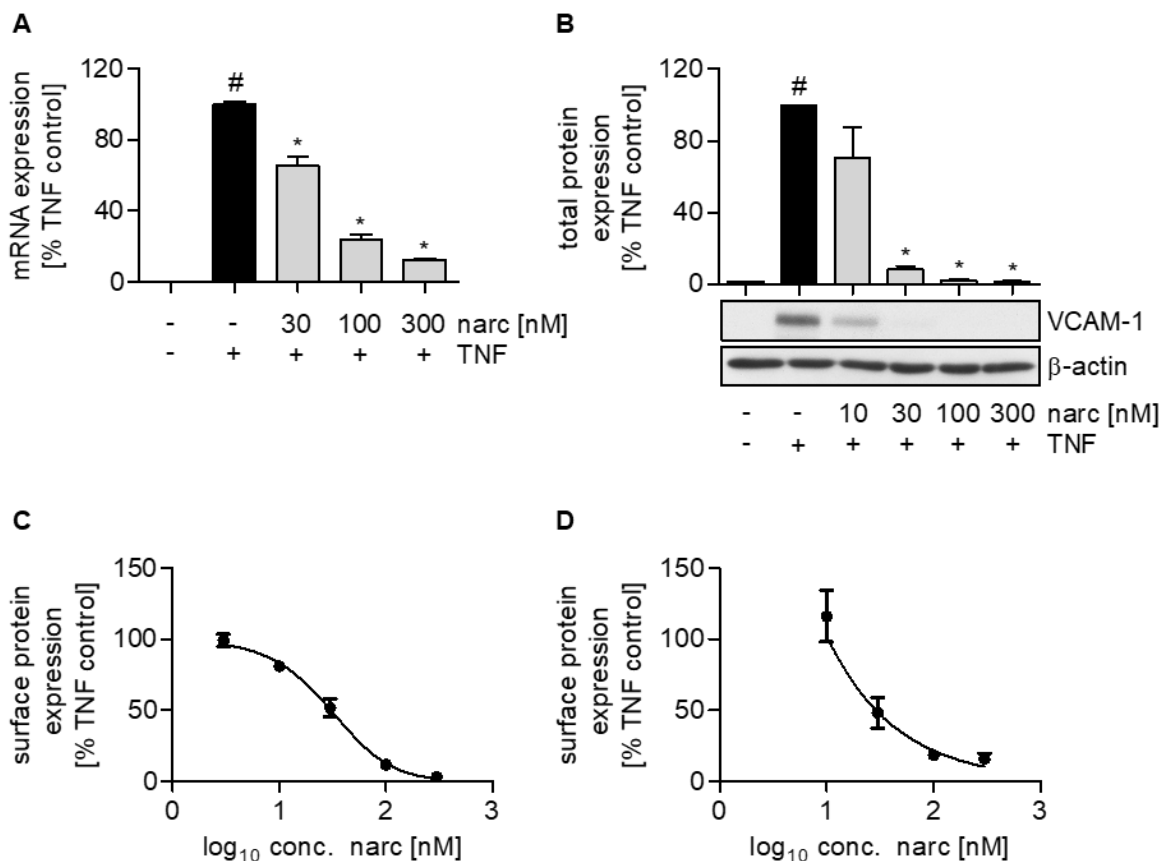
Figure 16: Narciclasine strongly reduces the TNF-caused VCAM-1 mRNA, total protein and surface protein expression in endothelial cells.
HUVEC were pre-treated with narciclasine (**(A):** 30, 100, 300 nM; **(B):** 10, 30, 100, 300 nM) for 30 min and then treated with 10 ng/ml TNF for **(A)** 12 h or **(B)** 24 h. **(A)** mRNA expression was analyzed with qPCR. **(B)** Protein expression levels were analyzed by western blotting. One representative blot out of three independently performed experiments is shown. Quantification was performed with ImageJ. **(A/B)** Data are expressed as mean ± SEM; n=3; *p ≤ 0.05 vs. TNF control, #p ≤ 0.05 vs. negative control. **(C)** Confluent HUVEC were pre-treated with narciclasine (3, 10, 30, 100, 300 nM)

for 30 min and then stimulated with 10 ng/ml TNF for 24 h. **(D)** Confluent hCMEC/D3 were pre-treated with narciclasine (10, 30, 100, 300 nM) for 30 min and then stimulated with 10 ng/ml TNF for 24 h. **(C/D)** Surface protein expression was analyzed by flow cytometry. Data are expressed as mean ± SEM; n=3.

VCAM-1 total protein expression was also downregulated by narciclasine at a concentration of 30 nM. At this concentration VCAM-1 protein expression was only 8 % from the positive control values (TNF-only treated cells). VCAM-1 expression levels continued dropping to negative control levels when HUVECs were treated with 300 nM narciclasine **(Figure 16B)**.

Confluent HUVECs or hCMEC/D3 were pre-treated with narciclasine and then activated with TNF. After the incubation, mRNA expression in HUVEC was measured using quantitative PCR (qPCR), and total protein expression was analyzed by western blotting.

The effect of narciclasine on the TNF-induced VCAM-1 surface protein expression was tested in HUVECs and in hCMEC/D3 cell lines. Confluent HUVECs or hCMEC/D3 were pre-treated with narciclasine and then activated with TNF for 24 h **(Figure 16C, 16D)**. Flow cytometric analysis showed that narciclasine downregulates the TNF-triggered VCAM-1 surface protein expression in HUVECs with an IC_{50} value of 30 nM and in hCMEC/D3 with a value of 29 nM.

3.3.4 Narciclasine downregulates ICAM-1 mRNA, total protein and cell surface protein expression in TNF-activated endothelial cells

To identify if narciclasine influenced the ICAM-1 mRNA and total protein expression, HUVECs were pre-treated with narciclasine and then activated with TNF for 12 and 24 h, respectively. Quantitative PCR was used to measure ICAM-1 expression. Narciclasine downregulated the TNF-induced ICAM-1 mRNA expression starting from a concentration of 100 nM **(Figure 17A)**. ICAM-1 total protein expression was identified by western blot analysis. Narciclasine downregulated ICAM-1 total protein expression starting from 10 nM **(Figure 17B)**. The TNF-triggered ICAM-1 cell surface protein expression was previously shown by our group to be downregulated by narciclasine in HUVECs.[142] In this project, to test the influence of narciclasine on the ICAM-1 surface protein expression the

hCMEC/D3 cell line was used. Confluent hCMEC/D3 were pre-treated with narciclasine and then stimulated with TNF for 24 h. Surface protein expression was identified by flow cytometry. Narciclasine downregulated the ICAM-1 surface protein expression induced by TNF in hCMEC/D3 with an IC_{50} value of 42 nM **(Figure 17C)**.
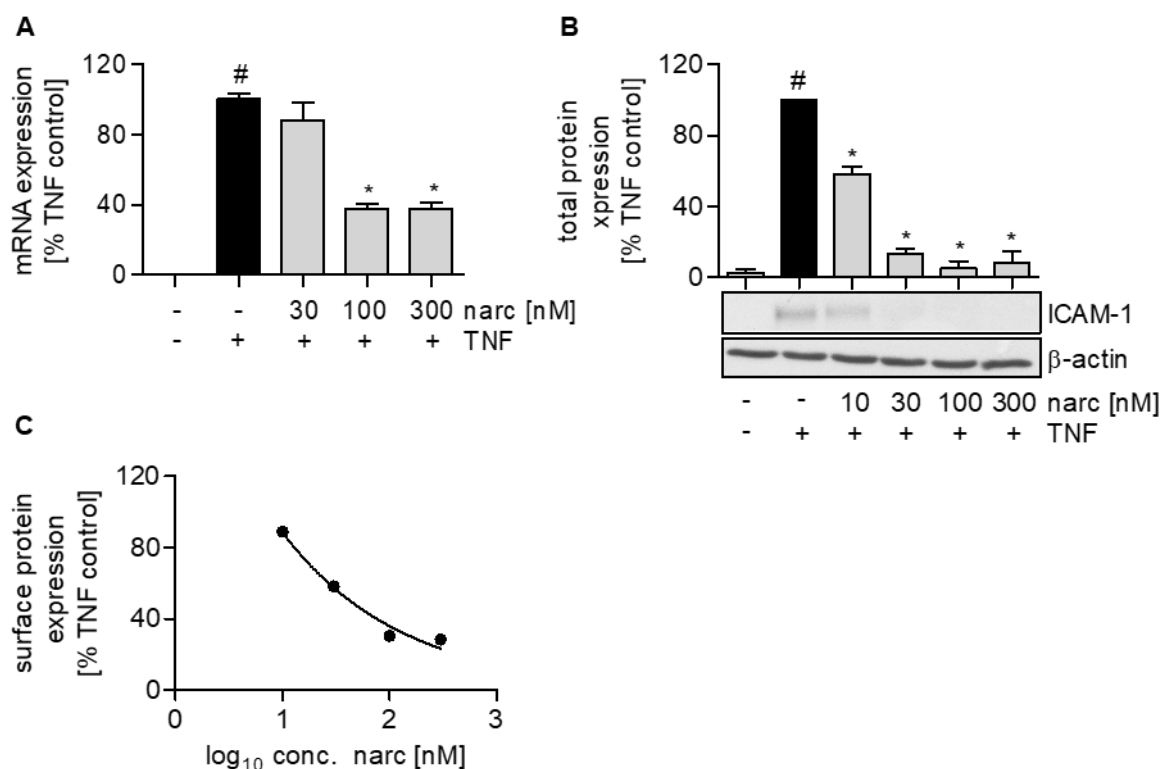

Figure 17: Narciclasine strongly reduces ICAM-1 mRNA, total protein and surface protein expression induced by TNF in endothelial cells.
(A/B) HUVEC were incubated with narciclasine for 30 min and then treated with 10 ng/ml TNF for **(A)** 12 h or **(B/C)** 24 h. **(A)** mRNA expression was analyzed with by qPCR. **(B)** Protein expression levels were analyzed by western blotting. One representative blot out of three independently performed experiments is shown. Quantification was performed with ImageJ. **(A/B)** Data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* TNF control, #p ≤ 0.05 *vs.* negative control. **(C)** Confluent hCMEC/D3 were pre-treated with narciclasine (10, 30, 100, 300 nM) for 30 min and then activated with TNF (10 ng/ml). After 24 h, surface protein expression was analyzed by flow cytometry. Data are expressed as mean ± SEM; n=3.

3.3.5 Narciclasine downregulates the TNF-induced expression of E-selectin mRNA, total and cell surface protein in endothelial cells

To determine the effect of narciclasine on the mRNA expression of E-selectin, confluent HUVEC were pre-treated with narciclasine for 30 min and then activated with TNF for 2 h. Results of the qPCR analysis showed that narciclasine at concentrations of 100 and 300 nM upregulated the TNF-induced mRNA expression of E-selectin mRNA **(Figure 18A)**. Total protein and cell surface expression was measured after the incubation of HUVECs with narciclasine for 30 min. Then TNF was added for 4 h. Western blot analysis revealed that the TNF-triggered total protein expression of E-selectin was significantly downregulated by narciclasine starting from 30 nM **(Figure 18B)**.
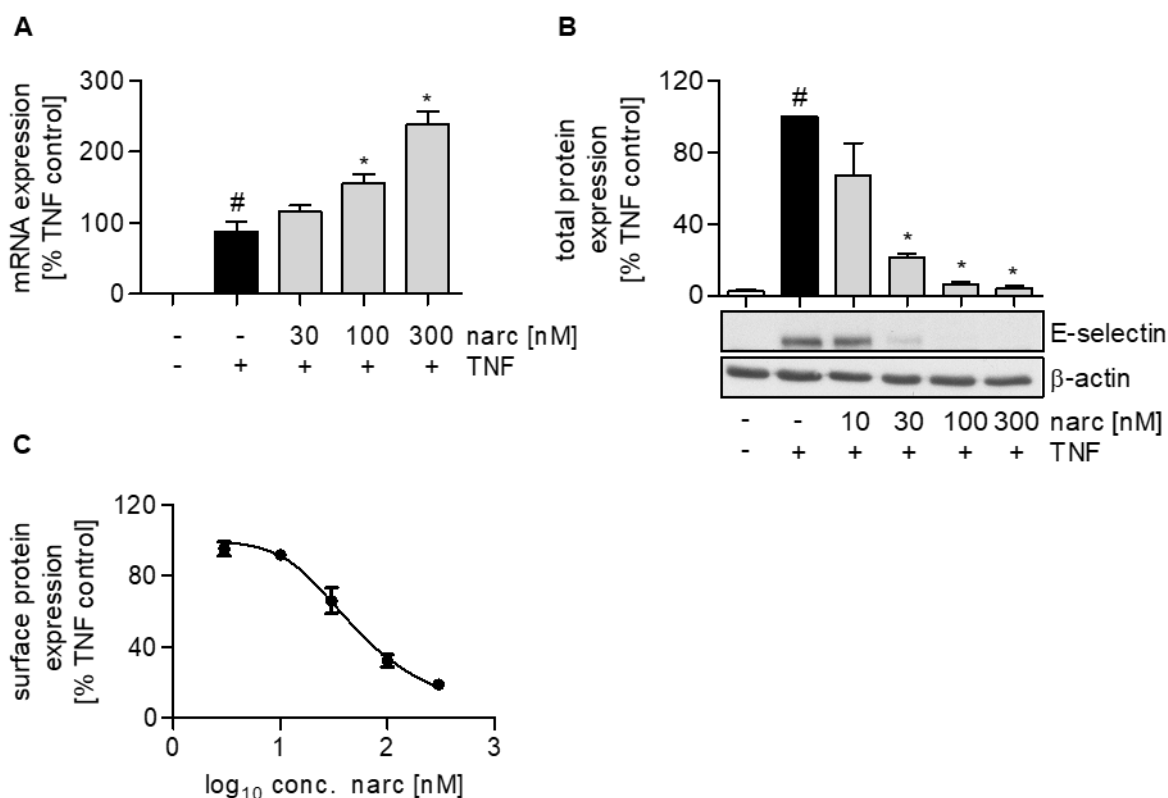
Figure 18: Narciclasine upregulates the TNF-induced E-selectin mRNA expression but downregulates its total protein and cell surface protein expression in HUVECs.
Confluent HUVECs were pre-treated with narciclasine for 30 min and then activated with 10 ng/ml TNF. **(A)** After 2 h of TNF activation mRNA expression was analyzed with qPCR. Narciclasine concentrations of 30, 100, 300 nM were used. **(B)** After 4 h, protein levels were analyzed by western blotting. One representative blot out of three independently performed experiments is shown.

Quantification was performed with ImageJ. Narciclasine was used at the concentrations 10, 30, 100 and 300 nM. **(A/B)** Data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* TNF control, #p ≤ 0.05 *vs.* negative control. **(C)** After 4 h, surface protein expression was analyzed by flow cytometry. Narciclasine was used at the concentrations 3, 10, 30, 100 and 300 nM. Data are expressed as mean ± SEM; n=3.

In addition, the TNF-caused upregulation of the surface levels of E-selectin in HUVECs was downregulated by narciclasine with an IC_{50} value of 50 nM **(Figure 18C)**. Thus, even though E-selectin mRNA expression was upregulated by narciclasine in response to TNF, its TNF-induced total protein and cell surface protein expression were significantly downregulated by the compound.

3.3.6 Narciclasine downregulates CX3CL1 mRNA and total protein expression in TNF-activated HUVEC

The influence of narciclasine on the CX3CL1 mRNA and total protein expression was analyzed in HUVECs. Confluent HUVECs were pre-treated with narciclasine for 30 min and then activated with TNF. qPCR analysis revealed that narciclasine strongly downregulates the TNF-induced expression of CX3CL1 mRNA starting from a concentration of 30 nM (
Figure 19A). Total protein expression of CX3CL1 was analyzed by western blotting. Starting from 30 nM, narciclasine strongly downregulated the TNF-triggered CX3CL1 protein expression in HUVEC (
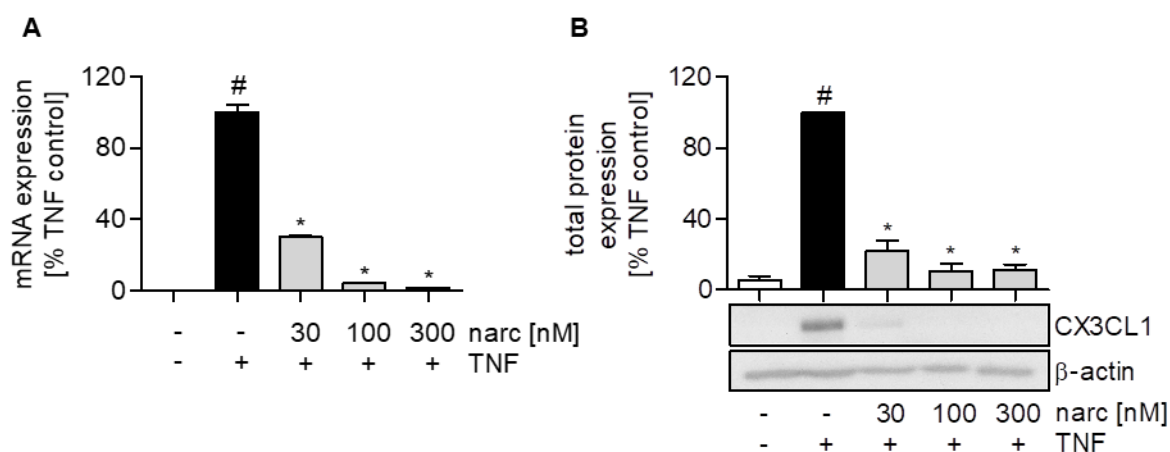**Figure 19B)**.

Figure 19: Narciclasine downregulates CX3CL1 mRNA and total protein expression in TNF-activated HUVECs.
Confluent HUVECs were pre-treated with narciclasine (30, 100, 300 nM) for 30 min and then activated with 10 ng/ml TNF for 12 h **(A)** or 24 h **(B)**. **(A)** mRNA expression was analyzed with qPCR. **(B)** Total protein expression levels were analyzed by western blotting. One representative blot out of three independently performed experiments is shown. Quantification was performed with

ImageJ. **(A/B)** Data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* TNF control, #p ≤ 0.05 *vs.* negative control.

3.4 Molecular mechanisms of the anti-inflammatory action of narciclasine

Narciclasine demonstrated the anti-inflammatory properties *in vivo* (**Figure 8**) and exerted an influenced on the interaction between leukocytes and endothelial cells *in vivo* **(Figure 9)** and *in vitro* **(Figure 13)**. Narciclasine also strongly downregulated the TNF-triggered cell surface protein expression of VCAM-1 **(Figure 16)**, ICAM-1 **(Figure 17)** and E-selectin **(Figure 18)** in endothelial cells. Total protein expression of CX3CL1, which serves as a strong attractant for leukocytes, was also downregulated by narciclasine upon TNF activation **(**
Figure 19). All these data strongly point out the beneficial influence of narciclasine on inflammation development.

The process of inflammation is regulated by a variety of mechanisms and signaling pathways within the cell. Trying to identify possible mechanisms of narciclasine's anti-inflammatory action we considered multiple pathways and proteins through which narciclasine exerts its anti-inflammatory properties. In the current chapter the influence of narciclasine on the following molecules and pathways will be demonstrated:

- cyclooxygenase-2 (COX 2);
- phosphorylated JNK and phosphorylated p38 (kinases of the MAP kinase pathway);
- Rho and Rho-kinase (ROCK);
- classical canonical NF-κB inflammatory pathway;
- TNF receptor 1 (TNFR1).

In the NF-κB activation cascade, the influence of narciclasine on the IκBα, IKK kinase, TAK-1 kinase and their phosphorylated forms were investigated. As TNF and IL-1 can trigger the canonical NF-κB cascade, the action of narciclasine on the protein expression of TNF receptor 1 and IL-1 receptor 1 was investigated as well.

3.4.1 Narciclasine upregulates COX2 mRNA and downregulates COX2 total protein expression in HUVEC

Cyclooxygenase-2 (COX2) is an enzyme, involved in the inflammatory response by taking part in the synthesis of various prostaglandins.[168, 169] Downregulation of COX2 was assumed to be a possible anti-inflammatory mechanism of action of narciclasine. To test this hypothesis mRNA and total protein expression levels of COX2 were analyzed in HUVECs. Briefly, confluent HUVECs were pre-treated with narciclasine for 30 min and then activated with TNF for 8 h to analyze mRNA expression or for 24 h to analyze total protein expression. qPCR analysis revealed that narciclasine did not affect the TNF-triggered expression of COX2 mRNA at concentrations of 10 to 100 nM, but further increased the expression at a concentration of 300 nM **(Figure 20A)**. Interestingly, an opposite effect was observed for the total protein expression. Narciclasine significantly downregulated the TNF-induced total protein expression of COX-2 at concentrations of 100 and 300 nM **(Figure 20B)**.
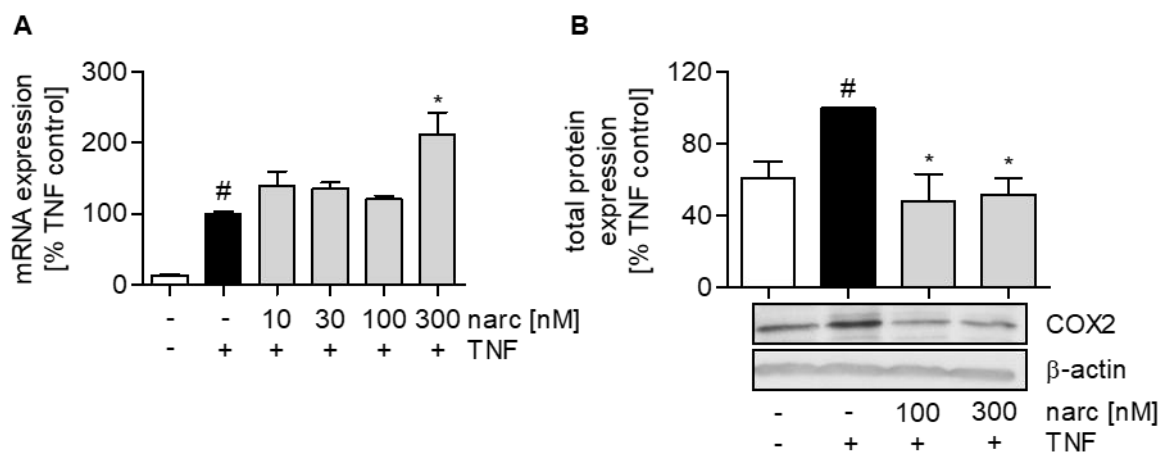

Figure 20: Narciclasine further upregulates the TNF-triggered COX2 mRNA expression at higher concentrations but downregulates its total protein expression in HUVECs.
(A) Confluent HUVECs were pre-treated with narciclasine (10, 30, 100, 300 nM) for 30 min and then activated with 10 ng/ml TNF for 8 h. mRNA expression was analyzed with qPCR. **(B)** Confluent HUVECs were pre-treated with narciclasine (100, 300 nM) for 30 min and then activated with 10 ng/ml TNF for 24 h. Total protein expression levels were analyzed by western blotting. One representative blot out of three independently performed experiments is shown. Quantification was performed with ImageJ. **(A/B)** Data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* TNF control, #p ≤ 0.05 *vs.* negative control.

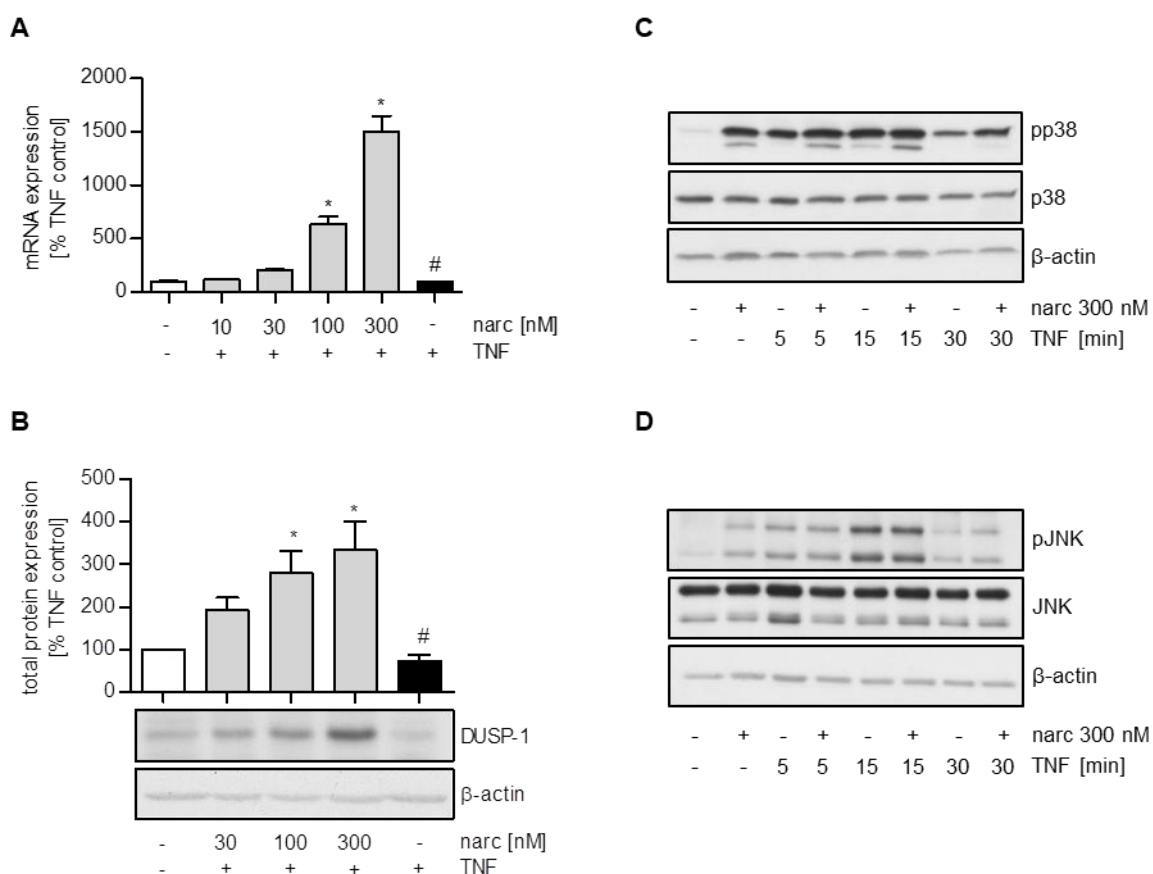## 3.4.2   Narciclasine upregulates DUSP-1 mRNA and total protein expression but does not influence the phosphorylation of p38 and JNK in HUVEC

The phosphorylation of the MAP kinases p38 and JNK, which contributes to inflammation development, is a key element in the activation of these kinases. The dual specificity protein phosphatase 1 (DUSP-1) is an enzyme, that blocks the phosphorylation of the p38 and JNK. Blocking of this phosphorylation reduces the activity of the kinases and stops signal transduction.[82, 84] We hypothesized that narciclasine might upregulate the expression of DUSP-1 and, thus, block the phosphorylation of p38 and JNK. First, it was tested if narciclasine influences DUSP-1 mRNA and total protein expression **(Figure 21A, 21B)**.

Figure 21: Narciclasine upregulates DUSP-1 mRNA and total protein expression, but it does not affect the TNF-induced phosphorylation of p38 and JNK in HUVECs.
(A/B) Confluent HUVECs were pre-treated with narciclasine for 30 min and then activated with 10 ng/ml TNF for 8 h. **(A)** mRNA expression was analyzed with qPCR. **(C/D)** Confluent HUVECs were pre-treated with narciclasine for 2 h and then activated with 10 ng/ml TNF for 5, 15 and 30 min. **(B/C/D)** Total protein expression was analyzed by western blotting. One representative blot out of two independently performed experiments is shown. **(A/B)** Data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* TNF control, #p ≤ 0.05 *vs.* negative control.

HUVECs were pre-treated with narciclasine and then activated with TNF for 8 h. Narciclasine strongly upregulated DUSP-1 mRNA **(Figure 21A)** and total protein expression **(Figure 21B)**. To investigate whether narciclasine blocks the phosphorylation of p38 and JNK, HUVEC were pre-treated with narciclasine for 2 h and then activated with TNF for 5, 15 and 30 min. Even though narciclasine strongly upregulated DUSP-1 total protein expression, the TNF-induced phosphorylation of p38 and JNK was not influenced **(Figure 21C, 21D)**.

3.4.3   The ability of narciclasine to downregulate ICAM-1 surface protein expression is not dependent on Rho

The small GTPase RhoA is responsible for non-muscle cell motility and contractility.[170-172] It has been shown that narciclasine activated Rho and, thus, stress fibers production, which lead to the increased survival rate in mice with glioblastoma.[132] However, to our knowledge, it has never been tested before if Rho could play a role in narciclasine's anti-inflammatory action. Thus, we tested if blocking of Rho and its effector kinase ROCK (Rho-associated protein kinase) diminishes the ability of narciclasine to downregulate cell surface levels of ICAM-1.

To perform the experiment, HUVECs were pre-treated with narciclasine (300 nM) for 30 min and either with an inhibitor of Rho (Rhosin, 10 µM) or an inhibitor of ROCK (Y-27632, 10 µM). Afterwards, cells were activated with TNF (10 ng/ml) for 24 h. ICAM-1 surface protein expression was analyzed with flow cytometry. Narciclasine downregulated the TNF-triggered increase of ICAM-1 surface protein expression even after ROCK **(Figure 22A)** or Rho **(Figure 22B)** were blocked by the inhibitors. The Rho inhibitor was also used in the cell adhesion assay. HUVECs were pre-treated with narciclasine (300 nM) for 30 min and then incubated with TNF (10 ng/ml) for 24 h. Together with narciclasine different concentration of the Rho inhibitor Rhosin were applied (1, 10, 20 µM). After 24 h, fluorescence-labeled Jurkat cells were allowed to adhere to a HUVEC monolayer for 1 h. Rho inhibition did not change the ability of narciclasine to reduce the TNF-caused adhesion of Jurkat cells to HUVECs **(Figure 22C)**.
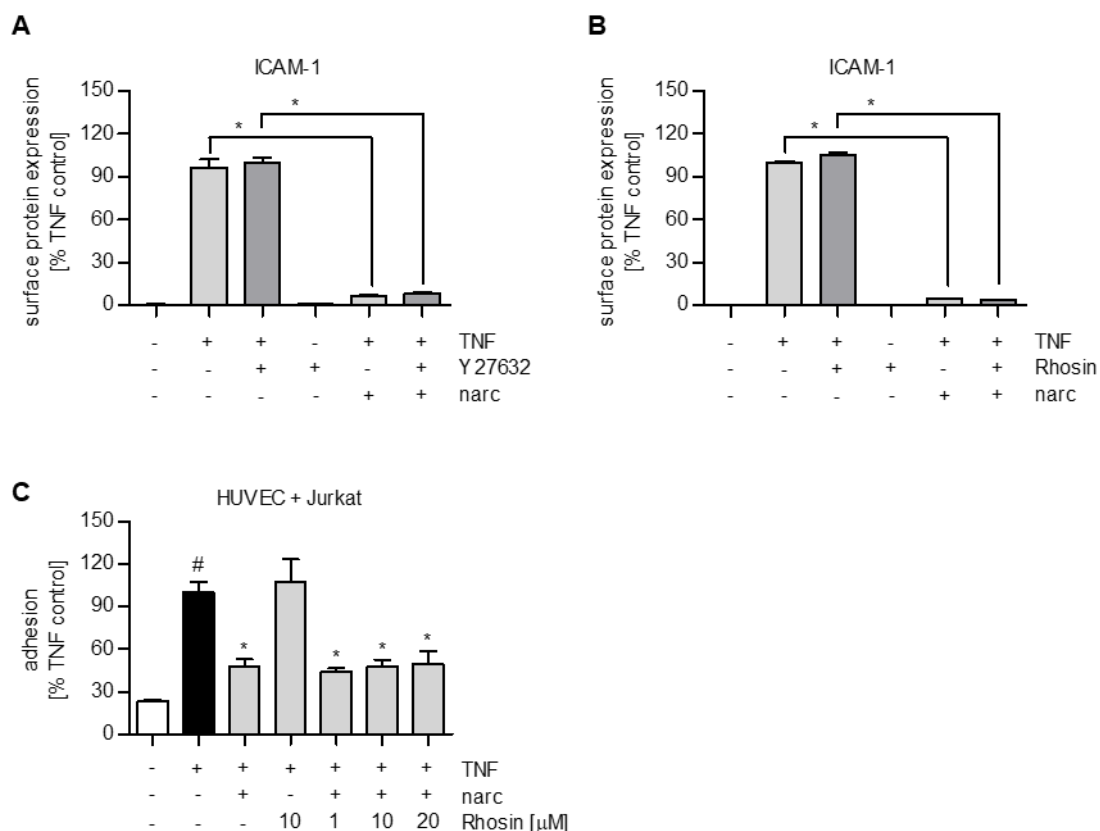
Figure 22: Rho and ROCK are not of importance for narciclasine's ability to downregulate ICAM-1 expression and Jurkat cells adhesion to HUVECs in response to TNF treatment. HUVECs were treated with narciclasine (300 nM) for 30 min and either an inhibitor of ROCK (Y-27632, 10 μM) **(A)** or an inhibitor of Rho (Rhosin: 10 μM **(B)**; 1, 10, 20 μM **(C)**). Then, TNF (10 ng/ml) was applied on HUVECs for 24 h. **(A/B)** ICAM-1 surface protein expression was analyzed by flow cytometry. **(C)** Fluorescently labeled Jurkat cells were allowed to adhere on the surface of a HUVECs monolayer for 1 h. **(A/B/C)** Data are expressed as mean ± SEM; n=3 **(A/B)**; n=2 **(C)**; *p ≤ 0.05 *vs.* TNF control **(A/B/C)**, #p ≤ 0.05 *vs.* TNF control **(C)**.

These data demonstrate that, neither Rho, nor its effector kinase ROCK contribute to narciclasine's ability to reduce the TNF-triggered interaction between leukocytes and endothelial cells.

3.4.4 Influence of narciclasine on the TNF-induced NF-κB activation pathway

The NF-κB signaling cascade plays an important part in the response of endothelial cells to pro-inflammatory stimuli.[61] The expression of endothelial cell adhesion molecules is dependent on the NF-κB transcription factor.[48] In this part we

investigated the classic canonical NF-κB pathway as it can be triggered by TNF,[61] which was used as a pro-inflammatory stimulus in all previous experiments.

3.4.4.1 Narciclasine inhibits NF-κB promoter activity induced by TNF

NF-κB transcription factor initiates transcription by connecting to the NF-κB dependent promoter on the genes, as a response to stimulation with pro-inflammatory cytokines, such as TNF. To elucidate molecular mechanisms responsible for narciclasine's anti-inflammatory properties, we investigated its influence on TNF-triggered NF-κB promoter activity using Dual-Luciferase Reporter Gene Assay. Narciclasine reduces NF-κB promoter activity, induced by TNF (10 ng/ml) starting at concentration of 100 and 300 nM **(Figure 23)**.
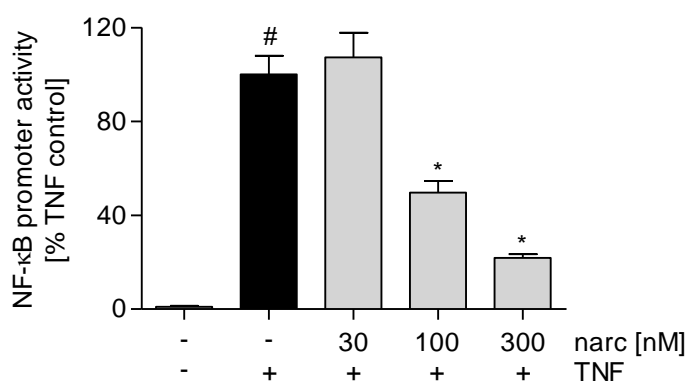
Figure 23: Narciclasine inhibits the activity of a TNF-induced artificial NF-κB promoter.
HUVECs were co-transfected with two vectors: 4.32 µg of pGL4.32[luc2P/NFκB-RE/Hygro] vector, coding for NF-κB response that regulate the expression of Photinus luciferase and 1.93 µg of pGL4.74[hRluc/TK] vector, coding for Renilla luciferase. 24 h after the transfection, HUVECs were pre-treated with narciclasine (30, 100, 300 nM) for 30 min and activated with TNF (10 ng/ml) for 5 h. A Dual Luciferase reporter gene assay was performed. NF-κB-dependent luciferase activity was determined by luminescence measurement. Data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* TNF control, #p ≤ 0.05 *vs.* negative control.

3.4.4.2 Narciclasine inhibits the TNF-activated translocation of the p65 NF-κB subunit into the nucleus

When transcription factor binds to the specific response elements on the promoter of a gene, RNA polymerase gets recruited and transcription process starts.[173] NF-κB promoter activity was significantly reduced by narciclasine. Therefore, the translocation of the NF-κB p65 subunit from the cytosol into the nucleus was investigated using fluorescence microscopy (
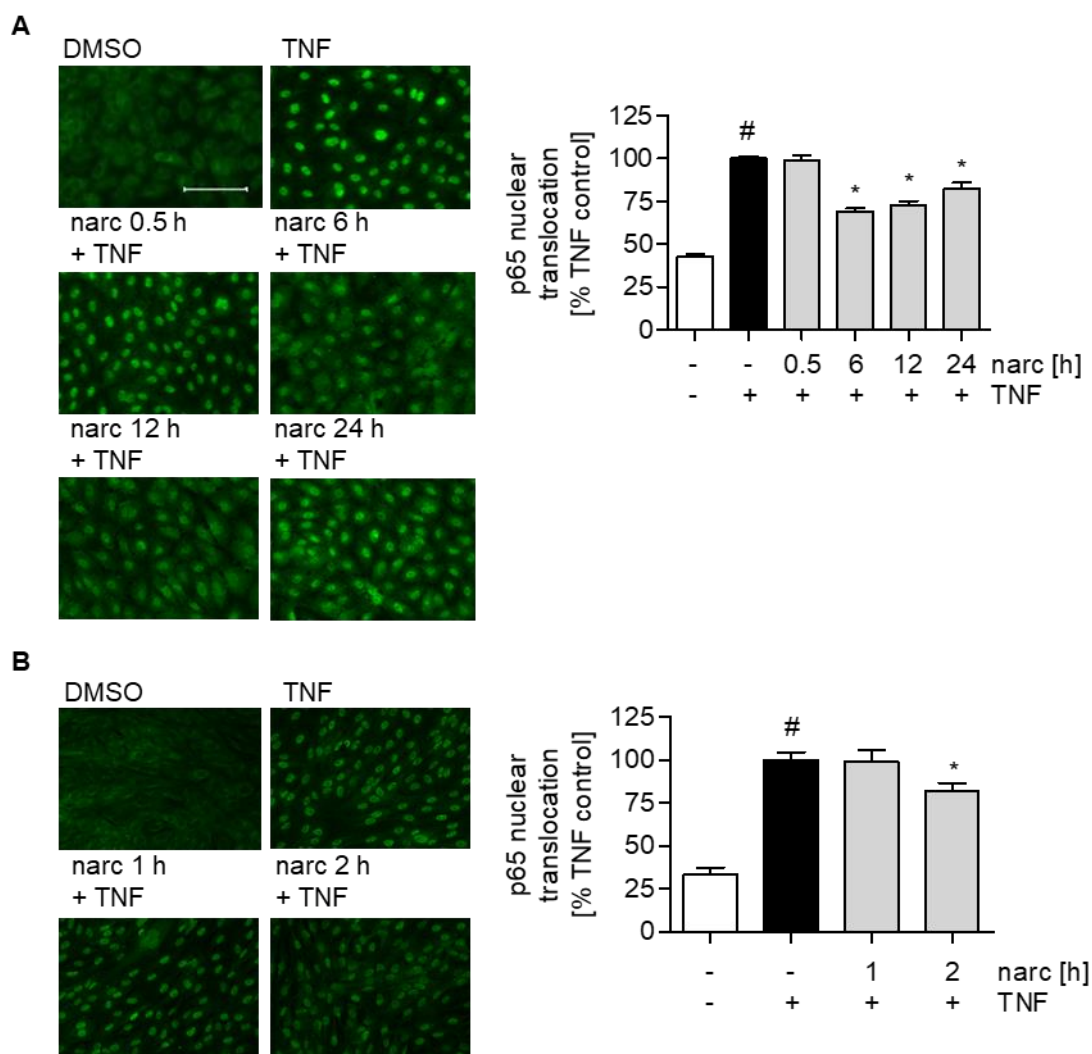).

Figure 24: Narciclasine inhibits the translocation of the p65 subunit of the NF-κB complex into the nucleus.
HUVECs were pre-treated with narciclasine (300 nM) for 0.5, 1, 2, 6, 12 and 24 h and activated with TNF (10 ng/ml) for 30 min. To detect p65, immunohistochemistry and fluorescence microscopy were performed. Scale bar represents 100 μm. One representative experiment out of three is shown. To obtain data for graphs **(A)** and **(B)** different HUVEC donors were used. Data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* TNF control, #p ≤ 0.05 *vs.* negative control.

Confluent HUVECs were pre-treated with narciclasine as indicated and then activated with TNF for 30 min. TNF-triggered nuclear translocation of p65 was

reduced already after 2 h of pretreatment with narciclasine (**Figure 23B**). This effect was still present after 24 h of pretreatment with the narciclasine (**Figure 23A, 23B**). Data for **Figure 24B** were obtained and analyzed by Dr. Rebecca Ingelfinger from the Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany.

3.4.4.3 Narciclasine inhibits the TNF-induced phosphorylation of IκBα

Under normal conditions, IκBα is bound to the NF-κB complex. During inflammation, IκBα gets phosphorylated and detaches from the NF-κB complex. Free IκBα gets degraded by the proteasomes within minutes.[67] As we have observed narciclasine reducing the TNF-triggered translocation of p65 from the cytosol into the nucleus (
), we decided to investigate its influence on the phosphorylation and degradation of IκBα, which is a prerequisite for the nuclear translocation of NF-κB.

In the following experiments, HUVECs were pre-incubated with narciclasine (300 nM) for 6 h before the application of TNF (10 ng/ml) for 5, 30 and 120 min. Afterwards, western blot analysis was performed. Narciclasine reduced the phosphorylation of IκBα induced by TNF at all tested time points **(Figure 25A)**. Narciclasine also prevented the early degradation of IκBα after 5 min of TNF **(Figure 25B)**. The recovery of IκBα after 30 min of TNF treatment was impaired by narciclasine. After 120 min of TNF treatment, narciclasine had no effect on IκBα **(Figure 25B)**.
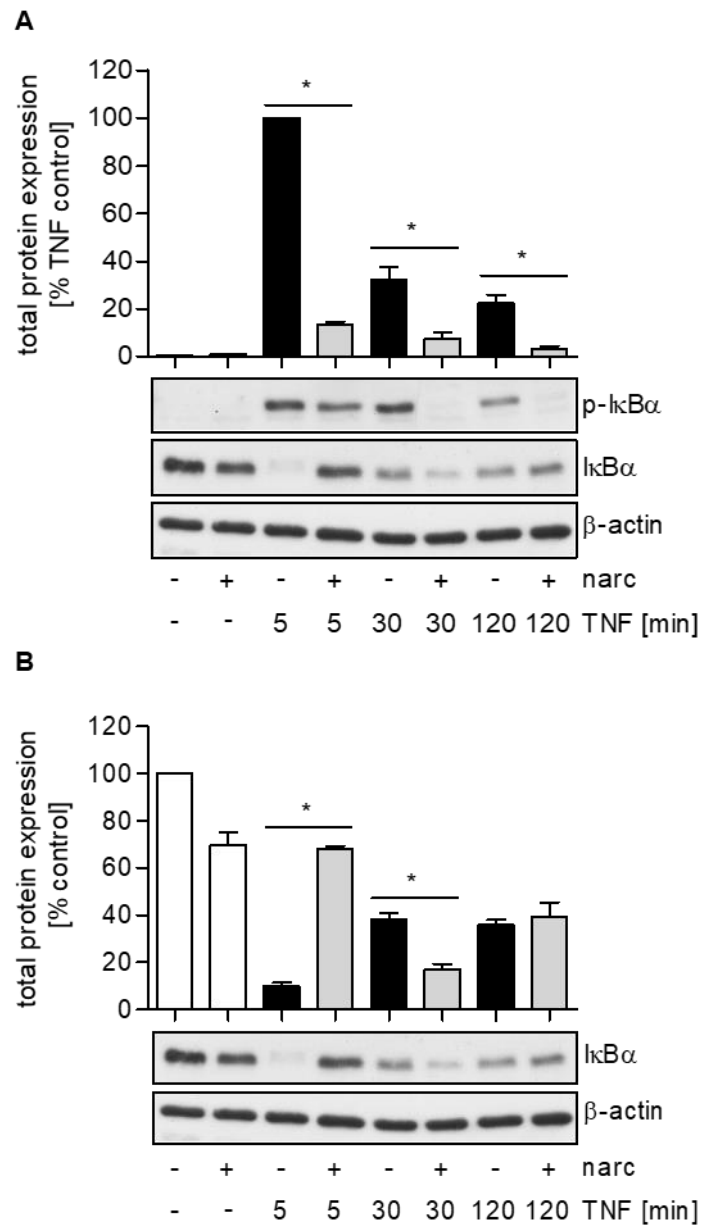
A

B

Figure 25: Narciclasine inhibits the TNF-induced phosphorylation of IκBα and impairs IκBα proteasomal degradation.
HUVECs were incubated with narciclasine (300 nM) for 6 h before treatment with TNF (10 ng/ml) for 5, 30 and 120 min. Western blot analysis was performed to identify protein expression levels of phosphorylated IκBα **(A)** and IκBα **(B)**. One representative western blot out of three is shown. For quantification data were normalized to β-actin levels. Quantified data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* negative control **(A)** or *vs.* TNF control **(B)**.

3.4.4.4   Narciclasine   inhibits   the   TNF-induced phosphorylation of IKKβ

The phosphorylation of IκBα was significantly reduced by narciclasine **(Figure 25A)**. This phosphorylation is dependent on the upstream kinase IκB kinase β (IKKβ).[67] To test the influence of narciclasine on IKKβ, HUVECs were pre-treated with narciclasine (300 nM) for 6 h and then incubated with TNF (10 ng/ml) for 5, 30 and 120 min. Then, western blot analysis of IKKβ phosphorylation was performed. The experiment demonstrated, that the TNF-induced phosphorylation of IKKβ was reduced by narciclasine already after 5 min of its induction with TNF and stayed on the same low level as the negative control for at least 2 h **(Figure 26)**.
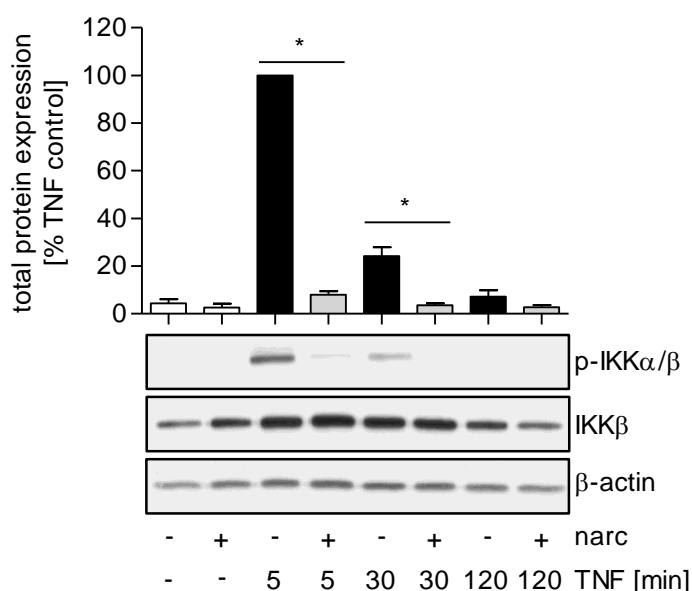

Figure 26: Narciclasine inhibits the TNF-induced phosphorylation of IKKβ.
Confluent HUVECs were treated with narciclasine (300 nM) for 6 h before stimulation with TNF (10 ng/ml) for 5, 30 and120 min. Phosphorylation of IKKβ was detected using western blot analysis. One representative western blot out of three is shown. For quantification data were normalized to β-actin levels. Quantified data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* TNF control.

3.4.4.5 Narciclasine inhibits the TNF-triggered phosphorylation of TAK-1

The phosphorylation of IKKβ is dependent on the upstream kinase TAK-1.[65] As the phosphorylation of IKKβ was strongly impaired by narciclasine **(Figure 26)**, the influence of narciclasine on the TAK-1 phosphorylation was analyzed. One out of three western blots were performed by Dr. Iris Bischoff-Kont from the Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany.

HUVECs were pre-treated with narciclasine (300 nM) for 6 h and then stimulated with TNF (10 ng/ml) for 5, 30 and 120 min. Then, western blot analysis revealed that narciclasine inhibited the TNF-induced TAK-1 phosphorylation **(Figure 27)**.
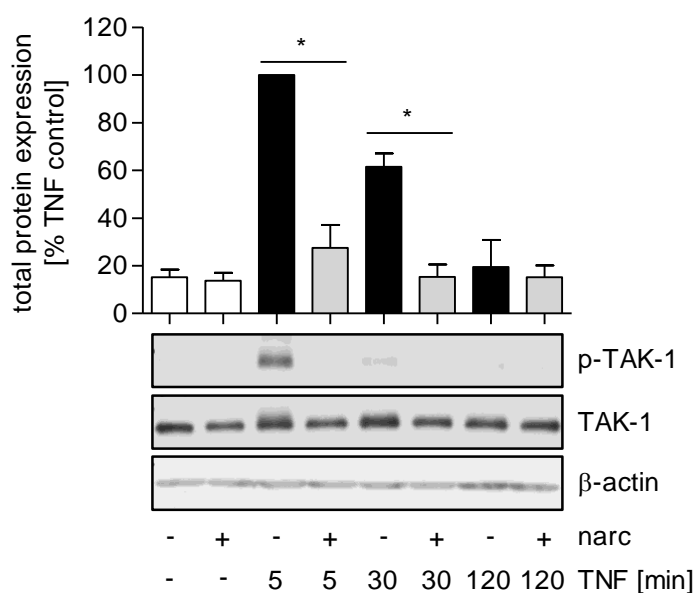

Figure 27: Narciclasine inhibits the TNF-induced phosphorylation of TAK-1.
HUVECs were first pre-treated with narciclasine (300 nM) for 6 h and then incubated with TNF (10 ng/ml) for 5, 30 and 120 min. Afterwards, western blot analysis was performed. One representative western blot out of three is shown. For quantification, data were normalized to β-actin levels. Quantified data are expressed as mean ± SEM; n=3; *$p \leq 0.05$ *vs.* TNF control.

3.4.5   Narciclasine inhibits the NF-κB activation cascade by decreasing the levels of the TNF receptor 1

In part **3.4.4** of this work it was demonstrated that narciclasine inhibits all crucial steps in the NF-κB activation cascade **(Figure 23-27)**. This led to the hypothesis that narciclasine could as well have an influence on the TNF receptor at the cell surface.

3.4.5.1   Narciclasine inhibits TNF receptor 1 expression on the cell surface of the endothelial cells

We tested if narciclasine has an influence on the expression of the main TNF receptor, the TNF receptor 1 (TNFR1). Confluent HUVECs were pre-treated with narciclasine (300 nM) for 6 h and then TNF (10 ng/ml) was added. Then, western blot analysis was performed to detect TNFR1 total protein levels. Narciclasine strongly reduced TNFR1 protein expression at all tested time points **(Figure 28A)**. It has also reduced TNFR1 protein expression when it was applied alone without subsequent treatment with TNF **(Figure 28A)**. This led to the hypothesis that narciclasine could act as an inhibitor of protein translation.

We compared the action of narciclasine to the action of the known translation inhibitor cycloheximide (CHX). For this, HUVECs were treated with narciclasine (300 nM) or CHX (10 µg/ml) for 0.5, 1, 2, 3 and 6 h. Narciclasine started its inhibitory action already after 1 h of treatment and lowered the levels of TNFR1 by approximately 60 % **(Figure 28B)**. The loss of TNFR1 after the treatment with narciclasine continued throughout all tested time points up to 6 h **(Figure 28B)**.

Next, it was tested if the proteasome has an influence on the narciclasine-triggered loss of TNFR1. This experiment was performed by Dr. Rebecca Ingelfinger and Dr. Iris Bischoff-Kont form the Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany. HUVECs were treated with both narciclasine and the proteasom inhibitor MG-132. As sown in **Figure 29**, narciclasine's ability to decrease TNFR1 levels was not signigicantly influenced by MG-132. Thus, it can be concluded that the loss of TNFR1 triggered by narciclasine is independent of the proteasome.
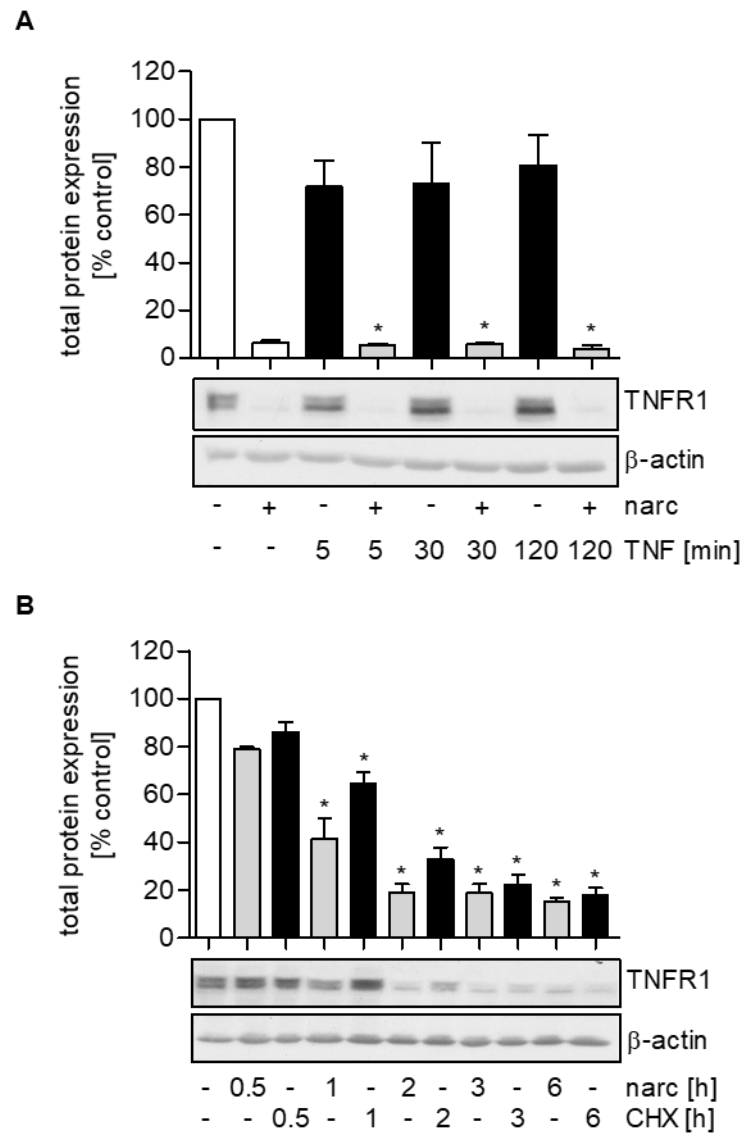
Figure 28: Narciclasine lowers TNF receptor 1 (TNFR1) levels.
HUVECs were treated with narciclasine (300 nM) for 6 h before treatment with **(A)** TNF (10 ng/ml) for 5, 30 and 120 min or **(B)** either with narciclasine (300 nM) or CHX (10 µg/ml) for 0.5, 1, 2, 3, 6 h. To identify TNFR1 total protein expression, western blot analysis was performed. One representative western blot out of three is shown. For quantification data were normalized to β-actin levels. Quantified data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* negative control.
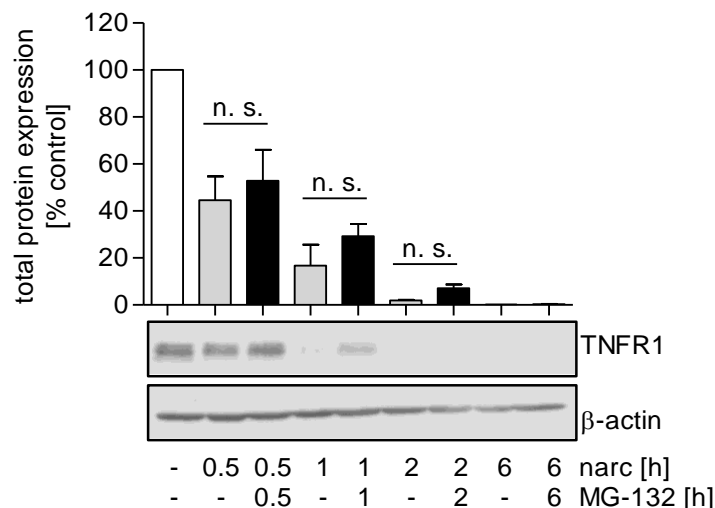
Figure 29: Narciclasine lowers TNF receptor 1 (TNFR1) levels independent of the proteasome.
HUVECs were treated with narciclasine alone (300 nM) or with a combination of narciclasine and the proteasome inhibitor MG-132 (10 µM) for 0.5, 1, 2, 6 h. TNFR1 total protein expression was detected using western blot analysis. One representative western blot out of three is shown. For quantification data were normalized to β-actin levels. Quantified data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* negative control.

3.4.5.2   Narciclasine does not influence the protein levels of IκBα, IKKβ and TAK-1

TNFR1 protein expression was strongly blocked by narciclasine, even when it was applied to the cells without TNF. Further experiments were performed to investigate if narciclasine had the same effect on other key players of the NF-κB activation cascade: IκBα, IKKβ and TAK-1. Therefore, HUVECs were treated with narciclasine (300 nM) for 5 min, 6 h, 8 h and 24 h. Then, western blot analysis was performed. Narciclasine had no effect on the total protein expression of IκBα, IKKβ and TAK-1 at any of the tested time points **(Figure 30A-C)**, but it blocked TNFR1 expression starting form 6 h treatment, and this effect was still present after 24 h **(Figure 30D)**.
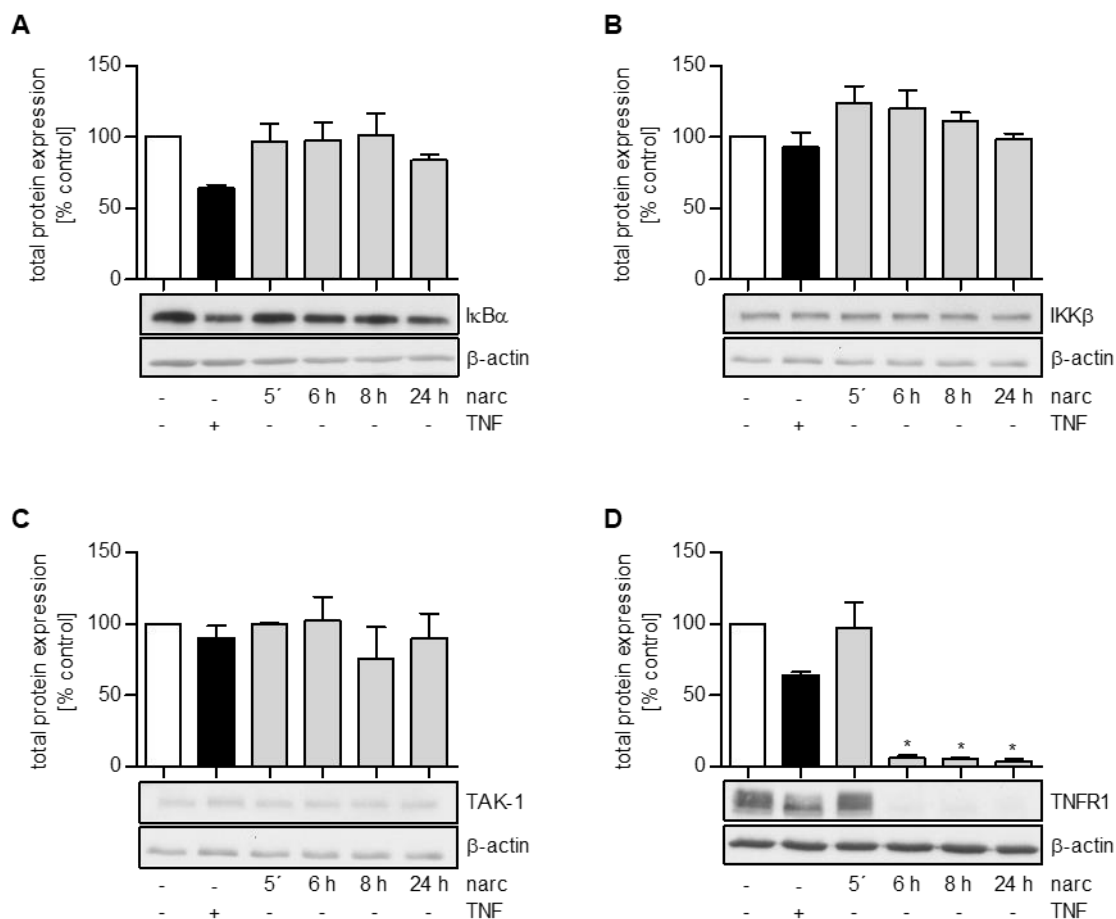
Figure 30: Narciclasine blocks TNF receptor 1 (TNFR1) expression, but does not reduce IκBα, IKKβ and TAK-1 total protein expression.
HUVECs were treated with narciclasine (300 nM) for 5 min, 6 h, 8 h and 24 h. As controls, cells were treated with TNF (10 ng/ml) or were left untreated for 24 h. Western blot analysis was performed to identify protein levels of IκBα **(A)**, IKKβ **(B)**, TAK-1 **(C)** and TNFR1 **(D)**. One representative western blot out of three is shown. For quantification data were normalized to β-actin levels. Quantified data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* negative control.

3.4.6 Narciclasine does not influence IL-1 receptor 1 protein levels

NF-κB signaling pathway can be triggered by TNF and IL-1.[61] As TNFR1 protein expression was blocked by narciclasine **(Figure 28, Figure 30D)**, it was interesting to investigate if it also influences the expression of the IL-1 receptor 1 (IL-1R1). HUVECs were treated with narciclasine (300 nM) for 5 min, 6 h, 8 h and 24 h. Western blot analysis revealed that narciclasine had no influence on the expression of the IL-1R1 **(Figure 31)**.
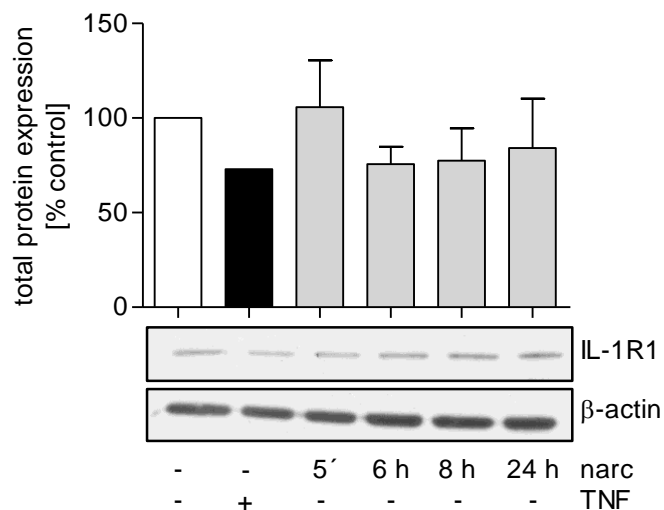
Figure 31: Narciclasine does not have an influence on IL-1 receptor 1 (IL1R1) expression.
Confluent HUVEC were treated with narciclasine (300 nM) for 5 min, 6 h, 8 h and 24 h or with TNF (10 ng/ml) for 24 h. To identify IL-1R1 protein expression, western blot analysis was performed. One representative western blot out of three is shown. For quantification, data were normalized to β-actin levels. Quantified data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* negative control.

4 DISCUSSION

4.1 Targeting leukocyte-endothelial cell inter-action as an approach to reduce inflammation

Narciclasine is an alkaloid derived from plants of *Narcissus species*. It has been widely recognized for its anti-cancer properties in many *in vitro* and *in vivo* experiments targeting various kinds of cancer as glioblastoma, melanoma and breast cancer.[129, 131, 136] However, two decades ago, it was shown that narciclasine has an anti-inflammatory potential. In 1999 Mikami *et al.* published a research where narciclasine showed promising anti-inflammatory effect in a rat model of arthritis.[137] Similar results were demonstrated by Luban *et al.* in 2012 also in a rat model of arthritis.[138] Luban and colleagues worked on the elucidation of narciclasine's anti-inflammatory mechanism. They showed that narciclasine reduced the expression of pro-inflammatory cytokines TNFα, IL-1β, IL-2 and IL-4 by PBMC cells.[138] These promising data became the basis for a deeper investigation of narciclasine as an anti-inflammatory compound.

Inflammation develops as a reaction to injury and plays a beneficial role in this regard. However, in autoimmune diseases inflammation occurs without a stimulation or damage coming as a threat from inside or outside of the organisms.[59, 174] The interaction between leukocytes and the endothelium is an important part of inflammation development.[44, 45] This interaction starts with the tethering of leukocytes. Then they start rolling on the surface of endothelial cells, comprising the blood vessels inner lining. The stimuli from leukocytes make endothelial cells express intracellular and vascular cell adhesion molecules (ICAM-1, VCAM-1), E-selectin and CX3CL1 on their surface. These molecules help leukocytes to adhere and subsequently, to transmigrate through the endothelial cell layer into the tissue where they cause inflammation.[48, 55]

Narciclasine represents a small molecule that interferes with the interaction between leukocytes and the endothelium. This was demonstrated *in vivo* and *in vitro*. Intravital microscopy revealed that the processes of leukocyte rolling, adhesion and transmigration to/through the endothelial cell layer were all found to be strongly inhibited by narciclasine **(Figure 9)**. It is known from the literature, that narciclasine impairs leukocytes activity [137, 138, 140] and the downregulation of the cell surface protein expression of ICAM-1 in endothelial cells.[142] Existing data

describes the influence of narciclasine separately on the leukocytes and endothelial cells. Our research is the first one where the effect of narciclasine on the interaction between leukocytes and the endothelium is demonstrated. These results round up the knowledge about narciclasine's action on leukocytes and endothelial cells reported so far.

In *in vitro* experiments, adhesion and transmigration of leukocytes were significantly reduced by narciclasine in response to TNF activation of endothelial cells **(Figure 12, Figure 13, Figure *14*,**

Figure 15). This correlates with the results of our *in vivo* findings. The adhesion of immune cells to endothelial cells in *in vitro* experiments was reduced by narciclasine independent of whether cell lines (THP-1, Jurkat) or if primary cells were used (PBMC, primary lymphocytes).

The majority of research has aimed to reveal the anti-inflammatory properties of narciclasine and focus on its the effects on immune cells.[137, 138, 140] The focus of this research is the action of narciclasine on endothelial cells. Adhesion of leukocytes to endothelial cells depends on cell adhesion molecules and CX3CL1. The protein expression of ICAM-1, VCAM-1, E-selectin and CX3CL1 was strongly downregulated by narciclasine in HUVECs **(Figure 16B, Figure 16C, Figure 17B,** Error! Reference source not found.**B,** Error! Reference source not found.**C,**
Figure 19C) as well as in hCMEC/D3 for ICAM-1 and VCAM-1 **(Figure 16D, Figure 17C)**. Narciclasine also effected the mRNA expression of these molecules by downregulating them, except for E-selectin. This is the first work where narciclasine's action on endothelial cells is demonstrated. In previous studies only different immune cells have been addressed. The exception is a study performed in our group where the surface protein expression of ICAM-1 was shown to be downregulated by narciclasine in response to the activation of HUVEC by TNF.[142]

It has already been recognized that targeting of the interaction between leukocytes and endothelium can be a promising approach to prevent unwanted inflammation.[58, 59, 175, 176] One of the most successful research in this area is connected to the discovery of natalizumab and vedolizumab. Natalizumab is a monoclonal antibody that binds to integrins on the surface of leukocytes.[177] As integrins are of importance for the interaction between leukocytes and endothelial cells, binding of natalizumab to leukocytes inhibits their ability to interact with endothelial cells. Natalizumab was shown to be effective for the treatment of multiple sclerosis.[178] Another example is vedolizumab, which has the same mechanism of action as natalizumab. Vedolizumab has been used in the treatment

of inflammatory bowel disease.[179-181] These monoclonal antibodies successfully impair the interaction between leukocytes and endothelial cells. However, various side effects and the high costs are still limitations for modern biopharmaceuticals. Application of narciclasine inhibits interaction between leukocytes and endothelial cells. Further research is needed to demonstrate if narciclasine has beneficial effect in *in vivo* models of multiple sclerosis and inflammatory bowel diseases as natalizumab and vedolizumab have. Of course, the detailed investigation of potential side effects must be performed as well.

4.2 Anti-inflammatory effects of narciclasine in the *in vivo* model of acute peritonitis

As a model for acute inflammation, the murine model of zymosan-induced peritonitis was chosen. This is a well-established model in the acute pain research.[158-160] It allows to identify pain caused to mice by measuring the dynamic weight bearing. When in pain, mice tend to put more weight on the front paws then on the hind paws. During experiments, peritoneal lavage can be collected, and the amount of immune cells that migrated into the peritoneal cavity can be measured as well as the concentration of the cytokines in the lavage.

Narciclasine in a dose of 1 mg/kg significantly reduced such hallmarks of inflammation such as: pain caused by inflammation, the infiltration of the immune cells to the site of inflammation and the expression of pro-inflammatory cytokines **(Figure 8)**. The number of neutrophils and monocytes which migrated into the peritoneum was significantly reduced by narciclasine. In addition, the concentration of the pro-inflammatory cytokines TNF, IL-6 and IL-1β was reduced in peritoneal lavage after treatment with narciclasine compared to vehicle treatment. These findings correlate with and completes the *in vitro* research, which indicated that narciclasine reduces cytokines production by immune cells.[138, 140, 141] Narciclasine demonstrated similar inhibitory effects on the levels of TNF, IL-6 and IL-1β in neonatal rats with sepsis.[182]

Thus, this *in vivo* zymosan-induced acute peritonitis model clearly shows narciclasine's anti-inflammatory activity. Until now *in vivo* experiments

demonstrating narciclasine's anti-inflammatory activity have been performed only by four working groups around the world.[137-139, 182]

Another interesting finding from this *in vivo* assay was that narciclasine reduced visceral inflammatory pain. This ability has been demonstrated in narciclasine for the first time. The search for compounds that can effectively reduce acute pain caused by inflammation is an ongoing process.[183-185] The outcomes of chronic pain reported from patients suffering from rheumatoid arthritis show that it significantly influenced both their emotional and physical conditions throughout their life span.[186, 187] Reduction of visceral pain was discovered as a new property of narciclasine during our research. Further investigation will need to be performed to identify whether narciclasine can reduce not only acute inflammation and pain, but also chronic inflammatory pain in, for example, in *in vivo* model of rheumatoid arthritis.

4.3 Mechanisms of narciclasine's anti-inflammatory action

Regarding narciclasine's anti-inflammatory action, only immune cells have been investigated in detail so far.[137, 138, 140, 141] In our research, we focused on the identification of molecular mechanisms underlining narciclasine's anti-inflammatory activity in endothelial cells. As a model, human umbilical vein endothelial cells (HUVEC) were used. As an activator and inflammatory agent, TNF was used in all experiments.

To identify the mechanism of narciclasine's anti-inflammatory action, its influence on NF-κB pathway and two kinases involved in MAP kinase pathway (JKN and p38) was investigated. We also looked into influence of narciclasine on cyclooxygenase 2 (COX2) expression, involved in inflammation by catalyzing the production of prostaglandins.[168, 169] Furthermore the involvement of the small GTPase Ras homolog gene family member A (RhoA) in narciclasine's anti-inflammatory action was investigated.

4.3.1 Targeting of NF-κB pathway to reduce unwanted inflammation

NF-κB is a transcription factor that is activated in response to pro-inflammatory stimulation. It starts acting once it translocates from the cytoplasm into the nucleus. NF-κB is responsible for the activation of various genes, coding for proteins that are important for the development of inflammation. Among these genes there are also genes coding for ICAM-1, VCAM-1 and E-selectin.[69] We demonstrated that the gene expression of ICAM-1 and VCAM-1 was downregulated by narciclasine and the expression of these genes is NF-κB-dependent, we investigated if narciclasine had influence on the NF-κB translocation into the nucleus and other components of the activation cascade upstream of NF-κB.

First, we identified that narciclasine inhibited the activity of NF-κB promoter. **(Figure 23)**. Thus, the expression of the genes, dependent on this promoter were impaired. Furthermore we tested the pathway steps upstream of NF-κB and identified that each tested step of the NF-κB activation cascade was inhibited by narciclasine: the phosphorylation of TAK-1, IKK-β and IκBα, IκBα degradation and p65 nuclear translocation **(**
-Figure 27). The whole NF-κB pathway activation was blocked by narciclasine. As a result of this, the expression of the cell adhesion molecules on the surface of the endothelial cells was downregulated.

In the screening for anti-inflammatory drugs, molecules targeting the NF-κB pathway showed promising results.[72, 73] Based on data from 2010, there are around 700 small molecules that were characterized as inhibitors of NF-κB pathway activation.[188] Depending on their mode of action, they can be divided into small molecules that reduce IKK activity, block NF-κB nuclear translocation and DNA-binding, block proteasomal function, upregulate IκB and inhibit NF-κB activation by protein phosphatases.

The most well-known conventional anti-inflammatory drugs acting on the NF-κB pathway are glucocorticoids[75] and the NSAIDs aspirin and sodium salicylate[74]. Aspirin, sodium salicylate and curcumin act by inhibiting IKK activation.[74, 189] The anti-inflammatory properties of antioxidants, such as vitamins E and C, butylated hydroxyanisole, and N-acetylcysteine are based on NF-κB transcription inhibition by removing reactive oxygen species (ROS).[190] Without ROS, NF-κB cannot be activated.[191, 192] There are also natural compounds, e.g. flavonoids and resveratrol, which inhibit IKK activity.[193, 194]

The problem with using conventional NF-κB pathway inhibitors is that they usually have a broad spectrum of actions and affect other pathways. Also direct blocking of such cascade elements as, for instance IκBα, leads on the one hand to the inhibition of inflammation development, but on the other hand to apoptosis, as, for example, TNF causes inflammation and apoptosis at the same time.[195, 196]

There has been a number of research ongoing to develop and test specific NF-κB pathway inhibitors.[72] In this perspective, narciclasine has shown to be a promising candidate as it inhibits not just one element of the NF-κB pathway, but the whole cascade. Since all steps of the NF-κB activation cascade inside the cell were downregulated by narciclasine, next we investigated whether the anti-inflammatory effects of narciclasine were possibly mediated through the TNF receptor 1 on the endothelial cell surface.

4.3.2   Inhibition of the TNF receptor 1 protein synthesis

Narciclasine significantly inhibited TNFR1 expression already after 5 min of treatment with TNF **(Figure 28A)**. As to the mechanisms of this action, it appeared that TNFR1 protein synthesis was affected by narciclasine. In a setting where narciclasine was tested without the presence of TNF, TNFR1 protein was gone from the surface of endothelial cells already after 2 h of treatment. The receptor did not recover even after 24 h **(Figure 28, Figure 30)**. To investigate, whether narciclasine inhibits TNFR1 protein synthesis it was compared with the effect of the known inhibitor of the protein synthesis cycloheximide (CHX)[197, 198]. The effect of narciclasine on the TNFR1 levels was similar to the action of CHX **(Figure 28B)**. These data suggest hypothesis that first the inhibition of protein biosynthesis takes place, after which TNFR1 gets rapidly degraded. This degradation might occur *via* the lysosomal pathway[199, 200], but not *via* the proteasome. Against the proteasomal pathway speaks data obtained in **Figure 29**, where the effect on cells treated with narciclasine and the proteasomal inhibitor MG-132[201] is shown. The inhibitory action of narciclasine on the levels of TNFR1 was not affected by the proteasomal inhibitor. This correlates with the already reported ability of narciclasine to inhibit ribosomal protein synthesis in cancer cells. [127, 131]

The levels of TAK-1, IκBα and IKK-β were not affected by narciclasine after 24 h of treatment **(Figure 30)**. This can be explained by a short half-life of TNFR1 in comparison to the three mentioned proteins. This action on the TNFR1 explains completely the action of narciclasine on the downstream components of the NF-κB pathway and its anti-inflammatory effects.

Interestingly, narciclasine had no influence on IL-1 receptor 1 (IL-1R1) protein levels **(Figure 31)**. The author's suggestion is that the half-life of IL-1R1 is longer than the one of TNFR1. According to the currently available publications, the analysis of the half-life of these receptors has never been performed.

This research demonstrates for the first time that that TNFR1 is not present on the cell surface when narciclasine is applied. Thus, TNF cannot trigger the development of inflammation *via* NF-κB pathway. The strategies to combat inflammation by targeting TNF have been recognized to be a promising approach in the treatment of rheumatoid arthritis, Crohn's disease and plaque psoriasis.[202, 203]

4.3.3   Influence of narciclasine on DUSP-1 and phosphorylation of JNK and p38

Narciclasine significantly upregulated DUSP-1 mRNA and protein expression after treatment with TNF for 8 and 24 h, respectively **(Figure 21A, 21B)**. DUSP-1 blocks phosphorylation of p38 and JNK kinases, which participate in the MAP kinase pathway.[82-84] However, the phosphorylation of p38 and JNK was not influenced by narciclasine **(Figure 21C, 21D)**. Thus, even though narciclasine increases DUSP-1 gene and protein expression, it was not possible to identify any effects on the MAP kinase pathway connected to the increase in DUSP-1 protein levels. Further investigation of other elements of MAP kinase pathway is needed to exclude narciclasine's influence on this pathway.

4.3.4 Narciclasine reduces cyclooxygenase 2 (COX2) protein expression

Cyclooxygenase 2 (COX2) is known for its ability to participate in inflammatory processes.[204, 205] In experiments with narciclasine, COX2 gene expression was upregulated by narciclasine **(Figure 20A)**, but at the same time its protein expression was downregulated **(Figure 20B)**.

COX2 is an enzyme which takes part in the arachidonic acid metabolism and the synthesis of prostaglandins.[168, 169] Beside inflammation prostaglandins participate in a number of physiological functions. This is why constant blockage of prostaglandin production is not desired.[206] However, a short-term treatment with COX2 blockers has shown to have a positive anti-inflammatory effect in, for example, murine autoimmune arthritis model.[204, 207] Mode of action of approved anti-inflammatory drugs celecoxib, rofecoxib, etoricoxib, and valdecoxib is based on the inhibition of COX2.[208] However, rofecoxib and valdecoxib have been withdrawn by FDA from usage in 2004 and 2005 respectively. The reason was their damaging effect leading to the increased risk of stroke and heart attack.[209, 210]

Downregulation of COX2 protein expression may represent one more anti-inflammatory mechanism which is utilized by narciclasine. As the next step to validate this hypothesis, the influence of narciclasine on the production of prostaglandins needs to be studied. The initial experiments performed within the presented research did not show consistent results (data not shown). The investigation in this direction is a point for a future research.

4.3.5 Anti-inflammatory mechanism of narciclasine does not involve RhoA

During the earlier investigation of narciclasine in cancer research it has been identified that narciclasine inhibited the activation of the small GTPase Ras homolog gene family, member A (Rho A).[132] Rho GTPases participate in the regulation of the cytoskeleton organization and in gene transcription. Rho GTPases influence stress fiber formation and filopodial extensions to

lamellipodia.[170, 171] There is the evidence that the blockage of Rho kinase prevents inflammation in Crohn's disease, experimental colitis and rheumatoid arthritis due to NF-κB inhibition.[211, 212]

In the presented study there was no influence on RhoA detected. Two experiments were performed: the first one was the measurement of ICAM-1 surface protein expression with the addition of inhibitors for RhoA and its kinase ROCK **(Figure 22A, 22B)**, in the second one cell adhesion assays with a RhoA inhibitor were performed **(Figure 22C)**. In both experiments narciclasine and TNF were added. The outcome of the two sets of experiments showed that the blockage of both RhoA and its kinase did not reverse the action of narciclasine on the TNF-treated HUVEC. The downregulation of ICAM-1 protein expression and the adhesion of Jurkat to HUVEC monolayer stayed on the same level as before the treatment with RhoA and ROCK inhibitors, leading to the conclusion that narciclasine's anti-inflammatory action does not involve RhoA.

4.4   Narciclasine's potentials as an anti-inflammatory compound

In this work, anti-inflammatory properties of narciclasine have been investigated. The hypothesis tested in this research was that these anti-inflammatory properties were based on the interference of narciclasine with the interaction between leukocytes and endothelial cells. This interaction is a crucial step in the extravasation of leukocytes to the site where they cause inflammation.[41, 44] Targeting this step has been recognized to be a promising approach in the reduction of unwanted inflammation.[59, 176]

Present research demonstrates narciclasine's potential to have anti-inflammatory activity. Firstly, anti-inflammatory properties of narciclasine were investigated in zymosan-induced peritonitis model. During these experiments it was demonstrated for the first time that narciclasine reduces inflammatory pain, concentration of pro-inflammatory cytokines TNF, IL-6, IL-1β and the presence of the neutrophils and monocytes in the peritoneal lavage **(Figure 8)**. We also investigated the influence of narciclasine on the leukocyte-endothelial cell interaction in *in vivo* and *in vitro*

experiments, and demonstrated its ability to significantly reduce leukocytes rolling, adhesion and transmigration to/through an endothelium activated by TNF **(Figure 9, Figure 12-**

Figure 15).

In the *in vitro* experiments we revealed that narciclasine reduces the expression of the molecules that are responsible for leukocytes adhesion to the endothelium (ICAM-1, VCAM-1, E-selection, CX3CL1), thus interfering with the interaction between immune cells and endothelial cells. We also performed *in vitro* experiments with the cell culture of human cerebral microvascular endothelial cells hCMEC/D3. When cells were treated with narciclasine and activated with TNF, narciclasine reduced VCAM-1 and ICAM-1 cell surface protein expression **(Figure 16D, Figure 17C)**. Further investigation of narciclasine's action on the cells mimicking BBB together with *in vivo* research using murine models of multiple sclerosis is needed to conclude if it has a beneficial effect in the course of multiple sclerosis.

Another part of our research included the investigation of the molecular mechanisms responsible for narciclasine's anti-inflammatory activity. Furthermore, the NF-κB signaling pathway was investigated in detail. We revealed that within the endothelial cell, narciclasine interferes with functioning of the classical inflammatory NF-κB pathway. It blocks TNF-induced NF-κB nuclear translocation and the degradation of IκBα and phosphorylation of the important kinases of the cascade (IKK-β, TAK-1) located upstream of NF-κB **(**
-Figure 27). This mode of action of narciclasine on endothelial cells was published for the first time by our working group in August 2019.[213] Most of the previous research was focused on narciclasine's action on leukocytes. One of these projects from the South China University of Technology was running at the same time with ours and was published in March 2019. In this project, it was demonstrated that narciclasine blocks NF-κB pathway after LPS-induced inflammation in macrophages.[214] The two projects round up the knowledge on the *in vitro* action of narciclasine on both endothelial cells and leukocytes. In both cases, the NF-κB pathway is involved. Blocking of the NF-κB cascade can contribute to the beneficial reduction of inflammation.[74, 75, 188] However, NF-κB pathway inhibitors need to applied with caution. Expression of many genes connected to apoptosis, cell proliferation reaction to stress and many others are dependent on NF-κB transcription factor. Without normally functioning NF-κB cascade all these processes will be impaired as well.[215]

In our research we demonstrated that narciclasine not only prevents NF-κB pathway from activation and influenced all steps of the NF-κB activation cascade, but also, above all blocked the expression of the TNFR1 protein synthesis **(Figure 28, Figure 30).** This action of narciclasine explains the shutdown of the NF-κB pathway. Without TNFR1, inflammation development cannot occur *via* the NF-κB pathway, which is crucial for the expression of the cell adhesion molecules.

Thus, inhibition of TNFR-1 protein translation is a base for narciclasine's interference with the interaction between leukocytes and endothelial cells. This interaction is the hallmark of inflammation development. The interference with this interaction underlines the mode of anti-inflammatory action of monoclonal antibodies natalizumab and vedolizumab, which have been approved for use in patients. These antibodies bind directly to the cell adhesion molecules and with this block the interaction between leukocytes and the endothelial cells.[177-179] In this work, it was demonstrated that narciclasine also interfered with this interaction. But in case of narciclasine the base of this interference was based on TNFR-1 protein translation. Targeting TNF has been previously shown to be a successful approach to address diseases such as rheumatoid arthritis, Crohn's disease and plaque psoriasis.[202, 203]

Inflammatory autoimmune diseases are still a very big problem in modern society. Diseases like rheumatoid arthritis and multiple sclerosis dramatically reduce the quality of patients' life and may cause preliminary death in some cases. Because of these diseases countries around the world spend a lot of money on healthcare and medication.[216, 217] Available therapies reduce the symptoms' severity but do not causally treat complex autoimmune diseases.[36] As there is a link between inflammation and autoimmune diseases, the continuous search for active compounds with anti-inflammatory potential is an ongoing process. Narciclasine with its anti-inflammatory action could have a potential to treat these diseases. But it needs to be considered, that in the present work, narciclasine's anti-inflammatory potential has been demonstrated only in the models for acute inflammation. In the future, further investigation could demonstrate if narciclasine can efficiently reduce chronic inflammation as well. Also, further *in vitro* and *in vitro* experiments need to be performed to explore the dose which can be applied *in vivo* to be on one hand effective for treatment of unwanted inflammation, and on the other hand to not cause unwanted side effects.

The identification of other mechanisms, apart from the inhibition of TNFR1, which contribute to narciclasine's anti-inflammatory action requires further research. One of these mechanisms might be the inhibition of COX2 protein expression and production of prostaglandins connected to it. This and other hypothesizes requires further detailed investigation as future project for our working group.

Taken together, narciclasine represents a potent anti-inflammatory agent, which justifies future *in vitro* and *in vivo* research with the goal to fully explore the anti-inflammatory potential of narciclasine.

4.5 Summary

Chronic inflammation is considered to be a cause of the autoimmune diseases such as rheumatoid arthritis, Alzheimer's disease, multiple sclerosis, *etc*. The search for active compounds with anti-inflammatory properties, which could be potentially applied for an effective treatment of these diseases is still in the focus of research.

Narciclasine is derived from plants of *Narcissus* species. It has been recognized to have anti-cancer activity and was extensively investigated in this direction since 1970s.[125, 127, 136] However, its anti-inflammatory activity was demonstrated as well in *in vivo* arthritis models.[137, 138] As to the mode of this anti-inflammatory action, only the influence on leukocytes has been investigated so far.

In this work, narciclasine showed anti-inflammatory properties in the zymosan-induced *in vivo* model of acute peritonitis. It reduced the infiltration of monocytes and neutrophils to the abdomen and the concentration of the pro-inflammatory cytokines TNF, IL-6 and IL-1β. Together with this, narciclasine reduced acute visceral pain caused by zymosan injection. This is the first study demonstrating narciclasine's ability to reduce inflammatory pain.

Narciclasine interfered with leukocyte-endothelial cell interaction in both *in vivo* and *in vitro* models. With the help of intravital microscopy, it was possible to observe the reduced rolling, adhesion and transmigration of leukocytes in the vessels of a TNF-treated murine cremaster muscle with the help of narciclasine. This observation was confirmed in the *in vitro* models for adhesion and transmigration

where narciclasine reduced the level of leukocyte's interaction with HUVECs. This kind of action was demonstrated for narciclasine for the first time.

The mechanism of narciclasine's anti-inflammatory action is based on the inhibition of ICAM-1, VCAM-1, E-selection and CX3CL1 protein expression on the surface of endothelial cells **(Figure 32)**.
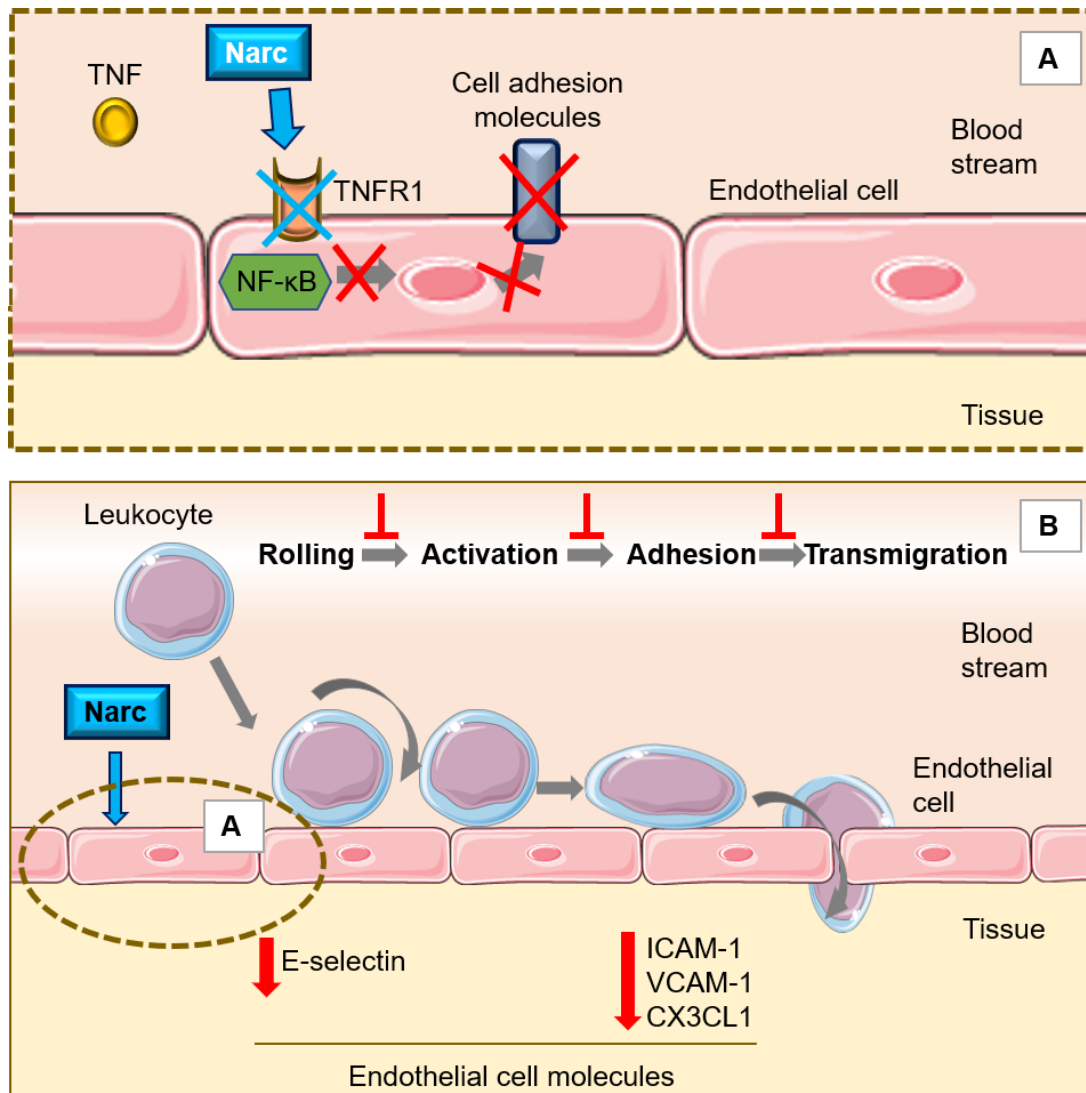

Figure 32: Narciclasine reduces inflammation by blocking TNFR1 protein synthesis and, thus, interfering with leukocyte-endothelial cell interaction.
(A) Narciclasine blocks protein synthesis of TNFR1. TNF cannot connect to its receptor on the endothelial cell surface and this blocks NF-κB activation cascade. Gene expression of the cell adhesion molecules (CAM) is dependent on the NF-κB transcription factor. Without NF-κB translocation to the nucleus, the gene expression of CAM is blocked. This prevents CAM expression on the endothelial cell surface. **(B)** Due to the downregulation of E-selectin, ICAM-1, VCAM-1 and CX3CL1 leukocytes rolling, adhesion and transmigration to/through the endothelial cell layer is impaired and thus, inflammation cannot start in the tissue. Pictures representing cells were taken from the online repository https://smart.servier.com/

The expression of ICAM-1, VCAM-1, E-selection and CX3CL1 on the cell surface is important for the adhesion of leukocytes to the endothelium. The molecular mechanism of the expression of these molecules is dependent on the NF-κB transcription factor. Narciclasine inhibited all steps of the NF-κB activation cascade by blocking protein synthesis of TNF receptor 1. Current work shows this mode of action in endothelial cells for the first time.

Narciclasine demonstrated profound anti-inflammatory properties based on its interference with leukocyte-endothelium interaction by downregulation of endothelial cell adhesion molecules expression and shutdown of NF-κB pathway. All these effects were a result of the TNF receptor 1 protein translation blocking by narciclasine. Without a receptor on the cell surface, TNF could not bind to the cell and start inflammatory cascade. These conclusions open a promising insight into the understanding of narciclasine's anti-inflammatory properties and justify further investigation of its potential for treatment of inflammatory diseases.

4.6 Conclusions and outlook

The results of this research expand the knowledge about narciclasine as an anti-inflammatory compound. These anti-inflammatory properties were highlighted in two *in vivo* experiments. Together with *in vivo* action, the molecular mechanisms of anti-inflammatory action were the key point of this research. We identified for the first time that in the context of anti-inflammatory properties narciclasine acts through blocking of TNFR1 protein synthesis. This inhibits the development of inflammation *via* NF-κB pathway and expression of cell adhesion molecules on the surface of endothelial cells. This connection between narciclasine's anti-inflammatory action and the underlying molecular mechanism has been demonstrated on the endothelial cells for the first time. The inhibition of NF-κB pathway is an established strategy against inflammation. In this regard, narciclasine presents itself as a compound with an effective mechanism to combat unwanted inflammation. This offers a good basis for further detailed research on anti-inflammatory properties of narciclasine.

We demonstrated that narciclasine downregulates the expression of the cell adhesion molecules and fractalkine. This happens due the blocking of TNFR1

expression, which prevents TNF from binding to its receptor on the cell surface. This is the basis for narciclasine's interference with the interaction between leukocytes and endothelial cells. In future work further *in vivo* and *in vitro* experiments need to be performed to get more insights into narciclasine's anti-inflammatory action.

Shut down of the NF-κB pathway through blocking of TNFR1 expression is an effective way to prevent the development of inflammation. However, this inhibition can have an effect of unwanted immune suppression and tissue damage. The inhibition of NF-κB pathway might not be the only cellular mechanism which is affected by narciclasine. As many other natural compounds narciclasine might operate through more than only one mechanism. In particular, it will be interesting to investigate in more detail narciclasine's ability to downregulate COX2 protein expression and connected to it reduction of prostaglandins production. Further experiments highlighting the action of narciclasine on prostaglandins levels will need to be performed.

One more protein, influenced by narciclasine was DUSP-1. Within MAP kinase pathway DUSP-1 dephosphorylates kinases p38 and JNK. Narciclasine upregulated DUSP-1 protein expression. However, we did not detect any influence of narciclasine on the phosphorylation of p38 and JNK kinases. Further research will need to be done to exclude that narciclasine does not interfere with other components of MAP kinase pathway.

Another direction, which would be interesting to investigate for understanding of further possible molecular mechanisms of narciclasine's action is looking into its possible blocking of binding of different chemokines to the cell surface. The first hint to this action was obtained in preliminary experiments, where narciclasine significantly downregulated the gene expression of Atypical Chemokine Receptor 1 (ACKR-1). ACKR-1 is a receptor for binding of 15 different chemokines. It is expressed on both endothelial cells and erythrocytes. In endothelial cells it takes part in transcytosis of tissue-derived chemokines and their presentation to circulation leukocytes. In our experiments already with 10 nM concentration of narciclasine the expression levels of ACKR-1 dropped 25 % compared to the TNF alone treatment **(Supplement Figure I)**. With 300 nM narciclasine concentration, the levels of ACKR-1 were reduced almost to the level of the negative control.

Narciclasine presents an active natural compound, which has a broad spectrum of properties. Its anti-cancer and anti-inflammatory activities have already drawn attention to this compound. However, we are just at the beginning of discovery of other potentially beneficial effects of narciclasine. There are definite indications that the spectrum of narciclasine's *in vivo* actions goes beyond anti-cancer and anti-inflammatory activity. In our research the compound has not only presented itself as an anti-inflammatory compound, but also reduced the inflammation-associated visceral pain in the zymosan-induced model of acute peritonitis. Further investigation needs to be done to assess whether narciclasine can reduce inflammation and pain not only under acute conditions, but also in the situation when inflammation is a chronical process. To sum up, narciclasine presents natural compound with strong anti-inflammatory properties. Its potential in treatment of the inflammatory autoimmune diseases is a subject for future investigation.

5 ZUSAMMENFASSUNG

5.1 Zusammenfassung

Eine Entzündung ist eine Reaktion des Immunsystems auf eine Verletzung des Gewebes oder auf Krankheitserreger. Bakterien, Viren, Pilze oder Parasiten werden als Krankheitserreger erkannt und durch das Immunsystem erfolgreich eliminiert. Eine Entzündung sollte nicht immer negativ betrachtet werden, da sie während einer Verletzung einen Beitrag zur Wundheilung leistet und somit hilft eine Infektion des ganzen Körpers zu verhindern. In diesen Fällen spielt die Entzündung eine wichtige Rolle und dient der Aufrechterhaltung der physiologischen Homöostase im Körper. In Fällen, in denen eine Entzündung ohne besonderen Grund erneut auftritt (z. B. Infektion oder Gewebeschädigung), spricht man von einer nichtphysiologischen Entzündung.

Nichtphysiologische Entzündungen sind teilweise für Autoimmunerkrankungen verantwortlich. Die eigenen Organe, Gewebe und Zellen des Körpers werden als feindseliges Ziel wahrgenommen und das Immunsystem reagiert mit der Ausbildung einer Entzündung. Es gibt auch einige Autoimmunerkrankungen, die durch andere Fehlfunktionen im Körper verursacht werden, sich jedoch als Entzündung manifestieren. Autoimmunerkrankungen sind in der modernen Gesellschaft nach wie vor ein großes Problem. Krankheiten wie rheumatoide Arthritis und Multiple Sklerose beeinträchtigen die Lebensqualität der Patienten dramatisch und führen in vielen Fällen zum vorzeitigen Tod. Obwohl die einzelnen Symptome und der Krankheitsverlauf mit Medikamenten gemindert werden sind diese Erkrankungen dennoch zum heutigen Stand nicht vollständig heilbar. Diese Krankheiten verursachen hohe Kosten für das Gesundheitssystem. Darüber hinaus können Nebenwirkungen den an chronischen Autoimmunerkrankungen leidenden Körper zusätzlich beeinträchtigen.

Eine wichtige Rolle bei einer Entzündungsreaktion spielen Endothelzellen und Leukozyten, welche im Blutkreislauf zirkulieren. Das Endothel, welches das Gefäßsystem von innen auskleidet, übernimmt eine entscheidende Funktion bei der Aufrechterhaltung der körpereigenen Homöostase. Endothelzellen werden durch bestimmte Zytokine aktiviert, die von Leukozyten sezerniert werden. Aktivierte Endothelzellen erleichtern die Migration der Leukozyten durch die Endothelbarriere des Blutgefäßes. Die Interaktion zwischen diesen Zelltypen

ermöglicht es den Leukozyten, den Blutkreislauf zu verlassen und in das Gewebe der Organe einzudringen, um dort eine Immunabwehr zu starten.

Einer der wichtigsten molekularen Mechanismen, der während einer Entzündung aktiviert ist, ist der NF-κB Signalweg. Sein kanonischer Teil, der bei der Entzündung eine Rolle spielt, kann durch IL-1 und TNFα ausgelöst werden. Durch eine Kaskade aus TRAF- und TAB-Proteinen wird die TAK-1-Kinase phosphoryliert. TAK-1 wiederum phosphoryliert die IKKβ Kinase, die sich im IKK-Komplex befindet. Der IKK-Komplex gilt als Hauptauslöser für die Aktivierung des NF-κB-Weges. Der IKK-Komplex besteht aus drei Untereinheiten: IKKα, IKKβ und NEMO/IKKγ. Während die genaue Rolle von NEMO noch unklar ist, phosphoryliert die Kinase IKKβ das Protein IκBα, einen Inhibitor von NF-κB. Diese Untereinheit ist Teil des NF-κB Komplexes. Dieser Komplex besteht aus IκBα inhibitorischen Untereinheiten (z.B. IκBα) und p65/RelA und p50/NF-κB Untereinheiten. Die Untereinheit IκBα hält den Transkriptionsfaktor NF-κB im Zytoplasma inaktiv und verhindert seine Translokation in den Zellkern. Wenn IκBα von IKKβ phosphoryliert wird, wird IκBα aus dem Komplex gelöst. Dies ermöglicht es Rel/NF-κB, in den Kern zu transportieren. Die Produktion einer Vielzahl von Molekülen, die für das Überleben, die Proliferation, die Entzündung und die Immunregulation wichtig sind, wird durch NF-κB ausgelöst. Die Hemmung der NF-κB-Aktivierungskaskade kann eine erfolgreiche Strategie zur Bekämpfung unerwünschter Entzündungen sein.

Derzeit gibt es Medikamente, die erfolgreich gegen Entzündungen eingesetzt werden. Viele bekannte Arzneimittel leiten sich von Naturstoffen ab. Naturstoffe sind Wirkstoffe, die in der Arzneimittelentwicklung eingesetzt werden und pflanzlichen, mikrobiellen oder tierischen Ursprungs sind. Heutzutage haben rund ein Viertel der Arzneimittel, die von der Food and Drug Administration (FDA) und / oder der European Medical Agency (EMA) zugelassen sind, im weitesten Sinne einen Naturstoff als Vorbild. Da bisher weniger als 10% der biologischen Vielfalt als potenzielle Quelle für bioaktive Verbindungen untersucht wurden, wird die Suche nach weiteren bioaktiven Substanzen fortgesetzt.

Unter den Naturprodukten, die aus Tieren, Pflanzen oder Mikroorganismen gewonnen werden, werden die aus Pflanzen gewonnenen Naturstoffe am längsten für medizinische Zwecke verwendet. Über die Verwendung von Pflanzen zur Behandlung einer Vielzahl von Krankheiten wurde bereits 2900 v. Chr. berichtet.

Mit Blick auf die Zukunft von pflanzlichen Naturprodukten werden sie weiterhin eine wertvolle Quelle für bioaktive Wirkstoffe sein. Im Laufe der Menschheitsgeschichte verbesserten sich die Techniken zur Extraktion und Reinigung von Wirkstoffen aus Pflanzen. Pflanzen wurden zu einer Quelle für viele Wirkstoffe, auf denen heute viele Medikamente basieren. Chinin und Artemisinin werden zum Beispiel in Arzneimitteln gegen Malaria eingesetzt. Sie werden aus den *Cinchona* spp. und *Artemisia annua* (Einjährige Beifuß) gewonnen. Krebsmedikamente wie Paclitaxel und Vinblastin sind ebenfalls pflanzlichen Ursprungs. Eines der bekanntesten Medikamente, das Morphin, wird aus dem Schlafmohn gewonnen (*Papaver somniferum*).

Ein weiterer interessanter bioaktiver Naturstoff, der pflanzlichen Ursprungs ist, ist Narciclasin. Er ist ein Alkaloid, das aus Pflanzen verschiedener Narzissen-arten gewonnen wird. Narciclasin wurde bereits intensiv auf seine krebshemmenden Eigenschaften hin untersucht und hat sich in vielen Tierversuchen, die verschiedene Krebsarten untersuchten, als ein vielversprechender Wirkstoff erwiesen. Vor zwei Jahrzehnten hat man damit begonnen, auch das entzündungshemmende Potenzial von Narciclasin zu untersuchen. Im Jahr 1999 veröffentlichten Mikami *et al.* eine Forschungsarbeit, bei der Narciclasin vielversprechende entzündungshemmende Eigenschaften in einem murinen Arthritis Modell zeigte. Ähnliche Ergebnisse konnten von Luban *et al.* 2012 ebenfalls in einem *In-vivo*-Arthritis-Modell erzielt werden. Um den Mechanismus dieser entzündungshemmenden Wirkung zu erklären, wurde der Einfluss von Narciclasin auf PBMCs (Peripheral Blood Mononuclear Cells; mononukleäre Zellen des peripheren Blutes) untersucht. Es konnte gezeigt werden, dass Narciclasin die Expression der proinflammatorischen Zytokine TNFα, IL-1β, IL-2 und IL-4 reduziert. Diese vielversprechenden Daten waren der Ausgangspunkt für eine tiefergehende Untersuchung von Narciclasin als ein entzündungshemmender Wirkstoff.

Die Interaktion zwischen den Leukozyten und Endothelzellen ist ein entscheidender Schritt bei der Migration der Leukozyten zu der Stelle, an der sie Entzündungen verursachen können. In dieser Arbeit wurde der Frage nachgegangen, ob Narciclasin diese Interaktion beinträchtigen kann.

Narciclasin zeigte entzündungshemmende Eigenschaften im Zymosan-induzierten Modell der akuten Peritonitis. Mit diesem Modell kann man durch Entzündung verursachte Schmerzen messen. Zusätzlich kann man die Menge der Immunzellen, welche ins Peritoneum kommen, zusammen mit Zytokinen, die als Reaktion auf Entzündung produziert werden, messen. In dieser Arbeit wurde es nachgewiesen, dass Narciclasin die Infiltration von Monozyten und Neutrophilen das Peritoneum und die Konzentration der proinflammatorischen Zytokine TNF, IL-6, IL-1β reduzierte. Ferner reduzierte Narciclasin akute viszerale Schmerzen, die durch die Peritonitis verursacht wurden. Somit konnte zum ersten Mal gezeigt werden, dass Narciclasin Entzündungsschmerzen reduzieren kann.

Narciclasin reduzierte die Interaktion von Leukozyten mit dem Endothel sowohl *in vitro* als auch *in vivo*. Mit Hilfe der intravitalen Mikroskopie konnte beobachtet werden, dass Narciclasin das Rollen, die Adhäsion und die Transmigration von Leukozyten in den Gefäßen reduziert hat. Diese Effekte wurden auch in den *In-vitro*-Modellen für Adhäsion und Transmigration beobachtet, in denen Narciclasin das Niveau der Interaktion von Leukozyten mit HUVECs (Human Umbilical Vein Endothelial Cells) reduziert hat. Diese Art von Wirkung wurde für Narciclasin zum ersten Mal gezeigt.

Der Mechanismus der entzündungshemmenden Wirkung von Narciclasin basiert auf der Hemmung der Expression von ICAM-1, VCAM-1, E-Selektin und CX3CL1-Proteinen auf der Oberfläche von Endothelzellen. Die Expression dieser Proteine ist wichtig für die Adhäsion von Leukozyten an das Endothel. Der molekulare Mechanismus der Expression dieser Moleküle ist abhängig vom Transkriptionsfaktor NF-κB. Narciclasin hemmte alle Schritte der NF-κB Aktivierungskaskade, indem es die Proteinsynthese des TNF-Rezeptors-1 blockierte. Diese Wirkungsweise wurde erstmals in Endothelzellen gezeigt.

Um andere mögliche Mechanismen zu identifizieren, die mit der entzündungshemmenden Wirkung von Narciclasin verbunden sind, ist es interessant, die Fähigkeit von Narciclasin, die COX2-Proteinexpression und die damit verbundene Verringerung der Prostaglandinproduktion genauer zu untersuchen. Weitere Experimente, die die Wirkung von Narciclasin auf den Prostaglandinspiegel aufzeigen, müssen noch durchgeführt werden.

Narciclasin ist ein Naturstoff mit einem breiten Eigenschaftsspektrum. Seine antitumoralen und entzündungshemmenden Wirkungen haben bereits die Aufmerksamkeit der Forschung auf sich gezogen. Wir stehen jedoch erst am Anfang der Entdeckung anderer potenziell nützlicher Wirkungen von Narciclasin. Es gibt eindeutige Hinweise darauf, dass das Spektrum der *In vivo*-Wirkungen von Narciclasin über die krebs- und entzündungshemmende Wirkung hinausgeht. In unserer Forschung präsentierte sich Narciclasin nicht nur als entzündungshemmende Verbindung, sondern reduzierte auch die entzündungsbedingten viszeralen Schmerzen im Zymosan-induzierten Modell der akuten Peritonitis. Weitere Untersuchungen müssen noch durchgeführt werden, um festzustellen, ob Narciclasin Entzündungen und Schmerzen nicht nur unter akuten Bedingungen, sondern auch in Situationen, in denen Entzündungen chronisch auftreten, lindern kann.

Die Ergebnisse dieser Forschung erweitern das Wissen über Narciclasin als entzündungshemmende Verbindung. Seine entzündungshemmenden Eigenschaften wurden in zwei *In-vivo*-Experimenten gezeigt. Ein wichtiger Bestandteil dieser Forschung ist die Untersuchung molekularer Mechanismen, welche für entzündungshemmende Wirkungen von Narciclasin verantwortlich sind. Wir haben zum ersten Mal nachgewiesen, dass entzündungshemmende Eigenschaften von Narciclasin durch die Blockierung der TNFR1-Proteinsynthese hervorgerufen werden. Dies hemmt die Entwicklung einer Entzündung über den NF-κB-Weg und die Expression von Zelladhäsionsmolekülen auf der Oberfläche von Endothelzellen. Der Zusammenhang von den entzündungshemmenden Eigenschaften und dem NF-κB-Weg wurde von uns erstmalig an Endothelzellen nachgewiesen. Die Hemmung des NF-κB-Signalwegs ist eine etablierte Strategie zur Bekämpfung von Entzündungen. In dieser Hinsicht präsentiert sich Narciclasin als Verbindung mit einem wirksamen Mechanismus zur Bekämpfung unerwünschter Entzündungen. Diese Schlussfolgerungen eröffnen einen vielversprechenden Einblick in das Verständnis der entzündungshemmenden Eigenschaften von Narciclasin und bieten eine gute Grundlage für weitere Untersuchungen seines Potenzials zur Behandlung entzündlicher Erkrankungen.

6 REFERENCES

References

1. His W. Die häute und höhlen des körpers. *Basel: Schwieghauser*. 1865
2. Florey H. The endothelial cell. *British Medical Journal* 1966;2:487-490
3. Pearce JA, Thomsen SL. Blood vessel architectural features and their effects on thermal phenomena. *Annu Rev Physiol* 2000;57:171-189
4. Augustin HG, Kozian DH, Johnson RC. Differentiation of endothelial cells: Analysis of the constitutive and activated endothelial cell phenotypes. *Bioessays*. 1994;16:901-906
5. Cahill PA, Redmond EM. Vascular endothelium - gatekeeper of vessel health. *Atherosclerosis*. 2016;248:97-109
6. Galley HF, Webster NR. Physiology of the endothelium. *British Journal of Anaesthesia*. 2004;93:105-113
7. Inagami T, Naruse M, Hoover R. Endothelium as an endocrine organ. *Annu Rev Physiol*. 1995;57:147-190
8. Esper RJ, Nordaby RA, Vilarino JO, Paragano A, Cacharron JL, Machado RA. Endothelial dysfunction: A comprehensive appraisal. *Cardiovasc Diabetol*. 2006;5:4
9. Shen Q, Wu MH, Yuan SY. Endothelial contractile cytoskeleton and microvascular permeability. *Cell Health Cytoskelet*. 2009;2009:43-50
10. Rubanyi GM. The role of endothelium in cardiovascular homeostasis and diseases. *Journal of cardiovascular pharmacology*. 1993;22 Suppl 4:S1-14
11. Michiels C. Endothelial cell functions. *J Cell Physiol*. 2003;196:430-443
12. Aird WC. Endothelial cell heterogeneity. *Cold Spring Harb Perspect Med*. 2012;2:a006429
13. Sandoo A, Veldhuijzen van Zanten JCS, Metsios GS, Caroll D, Kitas GD. The endothelium and its role in regulating vascular tone *The Open Cardiovascular Medicine Journal* 2010;4:302-312
14. Rajendran P, Rengarajan T, Thangavel J, Nishigaki Y, Sakthisekaran D, Sethi G, Nishigaki I. The vascular endothelium and human diseases. *Int J Biol Sci*. 2013;9:1057-1069
15. Libby P, Simon DI. Inflammation and thrombosis: The clot thickens. *Circulation*. 2001;103:1718-1720
16. Gori T, Burstein JM, Ahmed S, Miner SE, Al-Hesayen A, Kelly S, Parker JD. Folic acid prevents nitroglycerin-induced nitric oxide synthase dysfunction and nitrate tolerance: A human in vivo study. *Circulation*. 2001;104:1119-1123
17. Fuster V, Moreno PR, Fayad ZA, Corti R, Badimon JJ. Atherothrombosis and high-risk plaque: Part i: Evolving concepts. *Journal of the American College of Cardiology*. 2005;46:937-954
18. De Leeuw AM, Brouwer A, Knook DL. Sinusoidal endothelial cells of the liver: Fine structure and function in relation to age. *J Electron Microsc Tech*. 1990;14:218-236
19. Poisson J, Lemoinne S, Boulanger C, Durand F, Moreau R, Valla D, Rautou PE. Liver sinusoidal endothelial cells: Physiology and role in liver diseases. *J Hepatol*. 2017;66:212-227
20. Elvevold K, Smedsrod B, Martinez I. The liver sinusoidal endothelial cell: A cell type of controversial and confusing identity. *Am J Physiol Gastrointest Liver Physiol*. 2008;294:391-400

21. Stamatovic SM, Keep RF, Andjelkovic AV. Brain endothelial cell-cell junctions: How to "open" the blood brain barrier. *Current Neuropharmacology*. 2008;6:179-192

22. Daneman R, Prat A. The blood-brain barrier. *Cold Spring Harb Perspect Biol*. 2015;7:a020412

23. Claesson-Welsh L. Vascular permeability--the essentials. *Ups J Med Sci*. 2015;120:135-143

24. Sukriti S, Tauseef M, Yazbeck P, Mehta D. Mechanisms regulating endothelial permeability. *Pulm Circ*. 2014;4:535-551

25. Goodenough DA, Paul DL. Beyond the gap: Functions of unpaired connexon channels. *Nat Rev Mol Cell Biol*. 2003;4:285-294

26. Dejana E, Orsenigo F. Endothelial adherens junctions at a glance. *J Cell Sci*. 2013;126:2545-2549

27. Bazzoni G, Dejana E. Endothelial cell-to-cell junctions: Molecular organization and role in vascular homeostasis. *Physiol Rev*. 2004;84:869-901

28. Navarro P, Ruco L, Dejana E. Differential localization of ve- and n-cadherins in human endothelial cells: Ve-cadherin competes with n-cadherin for junctional localization. *The journal of Cell Biology*. 1998;140:1475-1484

29. Rubin LL. Endothelial cells: Adhesion and tight junctions. *Curr Opin Cell Biol*. 1992;4:830-833

30. Ivanov D, Philippova M, Antropova J, Gubaeva F, Iljinskaya O, Tararak E, Bochkov V, Erne P, Resink T, Tkachuk V. Expression of cell adhesion molecule t-cadherin in the human vasculature. *Histochemistry and cell biology*. 2001;115:231-242

31. Thoreson MA, Anastasiadis PZ, Daniel JM, Ireton RC, Wheelock MJ, Johnson KR, Hummingbird DK, Reynolds AB. Selective uncoupling of p120(ctn) from e-cadherin disrupts strong adhesion. *J Cell Biol*. 2000;148:189-202

32. Kotas ME, Medzhitov R. Homeostasis, inflammation, and disease susceptibility. *Cell*. 2015;160:816-827

33. Matzinger P. The danger model: A renewed sense of self. *Science*. 2002;296:301-305

34. Ashley NT, Weil ZM, Nelson RJ. Inflammation: Mechanisms, costs, and natural variation. *Annual Review of Ecology, Evolution, and Systematics*. 2012;43:385-406

35. Medzhitov R. Origin and physiological roles of inflammation. *Nature*. 2008;454:428-435

36. Scrivo R, Vasile M, Bartosiewicz I, Valesini G. Inflammation as "common soil" of the multifactorial diseases. *Autoimmun Rev*. 2011;10:369-374

37. Straub RH, Schradin C. Chronic inflammatory systemic diseases: An evolutionary trade-off between acutely beneficial but chronically harmful programs. *Evol Med Public Health*. 2016;2016:37-51

38. Roifman I, Beck PL, Anderson TJ, Eisenberg MJ, Genest J. Chronic inflammatory diseases and cardiovascular risk: A systematic review. *Can J Cardiol*. 2011;27:174-182

39. Amor S, Puentes F, Baker D, Van der Valk P. Inflammation in neurodegenerative diseases. *Immunology*. 2010;129:154-169

40. Rubio-Perez JM, Morillas-Ruiz JM. A review: Inflammatory process in alzheimer's disease, role of cytokines. *ScientificWorldJournal*. 2012;2012:756357

41. Netea MG, Balkwill F, Chonchol M, Cominelli F, Donath MY, Giamarellos-Bourboulis EJ, Golenbock D, Gresnigt MS, Heneka MT, Hoffman HM, Hotchkiss R, Joosten LAB, Kastner DL, Korte M, Latz E, Libby P, Mandrup-Poulsen T, Mantovani A, Mills KHG, Nowak KL, O'Neill LA, Pickkers P, van der Poll T, Ridker PM, Schalkwijk J, Schwartz DA, Siegmund B, Steer CJ, Tilg H, van der Meer JWM, van de Veerdonk FL, Dinarello CA. A guiding map for inflammation. *Nat Immunol*. 2017;18:826-831

42. Bonilla FA, Oettgen HC. Adaptive immunity. *J Allergy Clin Immunol*. 2010;125:S33-40

43. Kumar BV, Connors TJ, Farber DL. Human t cell development, localization, and function throughout life. *Immunity*. 2018;48:202-213

44. Middleton J, Patterson AM, Gardner L, Schmutz C, B.A. A. Leukocyte extravasation: Chemokine transport and presentation by the endothelium. *Blood*. 2002;100:3853-3860

45. Cook-Mills JM, Deem TL. Active participation of endothelial cells in inflammation. *J Leukoc Biol*. 2005;77:487-495

46. Muller WA. Getting leukocytes to the site of infmlammation *Vet Pathol*. 2013;50:7-22

47. Muller WA. Leukocyte-endothelial cell interactions in the inflammatroy response. *Laboratory Investigation*. 2002;82:521-533

48. Cronstein BN, Weissmann G. The adhesion molecules of inflammation *Arthitis and Rheumatism* 1993;36:147-157

49. Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone Jr MA. Identification of an inducible endothelial-leukocyte adhesion molecules. *Prot Natl Acad Sci USA*. 1987;84:9238-9242

50. Turowski P, Martinelli R, Crawford R, Wateridge D, Papageorgiou AP, Lampugnani MG, Gamp AC, Vestweber D, Adamson P, Dejana E, Greenwood J. Phosphorylation of vascular endothelial cadherin controls lymphocyte emigration. *J Cell Sci*. 2008;121:29-37

51. Cook-Mills MJ, Johnson JD, Deem TL, Ochi A, Wang L, Zheng Y. Calcium mobilization and rac1 activation are required for vcam-1 (vasular cell adhesion molecule-1) stimulation of nadph oxidase activity. *Biochem J*. 2004;378:539-547

52. Ley K, Laudanna C, Cybulsky MI, Nourchargh S. Getting to the sit eo finglammatio: The leukocyte adhesion cascade updated. *Nature Reviews Immunology*. 2007;7

53. Imaizumi T, Yoshida H, Satoh K. Regualiton of cx3cl1/fractalkine expression in endothelial cells. *J. Atheroscler Thromb*. 2003;11:15-21

54. Matsumiya T, Ota K, Imaizumi T, Yoshida H, Kimura H, Satoh K. Characterization of synergistic induction of cx3cl1/fractalkine by tnf-alpha and ifn-gamma in vascular endothelial cells: An essential role for tnf-alpha in post-transcriptional regulation of cx3cl1. *J Immunol*. 2010;184:4205-4214

55. Goda S, Imai T, Yoshie O, Yoneda O, Inoue H, Nagano Y, Okazaki T, Imai H, Bloom ET, Domae N, Umehara H. Cx3c-chemokine, fractalkine-enhanced adhesion of thp-1 cells to endothelial cells through integrin-dependent and -independent mechanisms. *J Immunol*. 2000;164:4313-4320

56. Umehara H, Bloom ET, Okazaki T, Nagano Y, Yoshie O, Imai T. Fractalkine in vascular biology: From basic research to clinical disease. *Arterioscler Thromb Vasc Biol*. 2004;24:34-40

57. Bjerkeli V, Damas JK, Fevang B, Holter JC, Aukrust P, Froland SS. Increased expression of fractalkine (cx3cl1) and its receptor, cx3cr1, in wegener's

granulomatosis--possible role in vascular inflammation. *Rheumatology (Oxford)*. 2007;46:1422-1427

58. Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: Present and future therapeutic targets. *Nat Immunol*. 2005;6:1182-1190

59. Ulbrich H, Eriksson EE, Lindbom L. Leukocyte and endothelial cell adhesion molecules as targets for therapeutic interventions in inflammatory disease. *Trends in Pharmacological Sciences*. 2003;24:640-647

60. Funk CD. Prostaglandins and leukotreins: Advances in ecosanoid biology. *Science*. 2016;294:1871-1875

61. Lawrence T. The nuclear factor nf-кb pathway in inflammation. *Cold Spring Harb Perspect Biol* 2009;1:a001615

62. Chandel NS, Trzyna WC, McClintock DS, Schumacker PT. Role of oxidants in nf-kappa b activation and tnf-alpha gene transcription induced by hypoxia and endotoxin. *J Immunol*. 2000;165:1013-1021

63. Frazier WJ, Xue J, Luce WA, Liu Y. Mapk signaling drives inflammation in lps-stimulated cardiomyocytes: The route of crosstalk to g-protein-coupled receptors. *PLoS One*. 2012;7:e50071

64. Kim HK. Role of erk/mapk signalling pathway in anti-inflammatory effects of ecklonia cava in activated human mast cell line-1 cells. *Asian Pacific Journal of Tropical Medicine*. 2014;7:703-708

65. Adhikari A, Xu M, Chen ZJ. Ubiquitin-mediated activation of tak1 and ikk. *Oncogene*. 2007;26:3214-3226

66. Israëli A. The ikk complex, a cnetral regulator of nf-кb activation. *Cold Spring Harb Perspect Biol*. 2010;2:a000158

67. Karin A, Delhase M. The iкb kinase (ikk) and nf-кb: Key elements of proinflamamtory signalling. *Seminars in Immunology*. 2000;12:85-98

68. Chen F, Casatova V, Shi X. New insights into the role of nuclear factor-кb in cell growth regulation. *American Joutnsl of Pathology*. 2001;159:387-397

69. Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: Nf-кb and cytokine-inducible enhancers. *FASEB J*. 1995;9:899-909

70. Hahn M, Macht A, Waisman A, Hovelmeyer N. Nf-kappab-inducing kinase is essential for b-cell maintenance in mice. *Eur J Immunol*. 2016;46:732-741

71. De Silva NS, Anderson MM, Carette A, Silva K, Heise N, Bhagat G, Klein U. Transcription factors of the alternative nf-kappab pathway are required for germinal center b-cell development. *Proc Natl Acad Sci U S A*. 2016;113:9063-9068

72. Makarov SS. Nf-кb as a therapeutic target in chrinic inflammation: Recent advances. *Molecular Medicine Today*. 2000;6:441-448

73. Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the nf-kappab pathway in the treatment of inflammation and cancer. *J Clin Invest*. 2001;107:135-142

74. Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of i(kappa)b kinase-beta. *Nature*. 1998;396:77-80

75. Karin M. New twists in gene regulation by glycocorticoids receptor: Is DNA binding dispensable? *Cell*. 1998;93

76. Plotnikov A, Zehorai E, Procaccia S, Seger R. The mapk cascades: Signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochim Biophys Acta*. 2011;1813:1619-1633

77. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by erk, jnk, and p38 protein kinases. *Science.* 2002;298:1911-1912

78. Yang D, Tournier C, Wysk M, Lu HT, Xu J, Davis RJ, Flavell RA. Targeted disruption of the mkk4 gene caues embryonic death, inhibition of c-jun nh2-terminated kinase activation, and defects in ap-1 transcription activity. *Proc Natl Acad Sci U S A.* 1997;94:3004-3009

79. Loewe R, Pillinger M, de Martin R, Mrowietz U, Groger M, Holnthoner W, Wolff K, Wiegrebe W, Jirovsky D, Petzelbauer P. Dimethylfumarate inhibits tumor-necrosis-factor-induced cd62e expression in an nf-kappa b-dependent manner. *J Invest Dermatol.* 2001;117:1363-1368

80. Jensen LE, Whitehead AS. Elam-1/e-selectin promoter contains an inducible ap-1/creb site and is not nf-кb-specific. *BioTechniques.* 2003;35:54-58

81. Kondoh K, Nishida E. Regulation of map kinases by map kinase phosphatases. *Biochim Biophys Acta.* 2007;1773:1227-1237

82. Li Q, Valerio MS, Kirkwood KL. Mapk usage in periodontal disease progression. *J Signal Transduct.* 2012;2012:308943

83. Patterson KI, Brummer T, O'Brien PM, Daly RJ. Dual-specificity phosphitases: Critical regulators with diverse cellular targets. *Biochem J.* 2009;418:475-489

84. Chi H, Barry SP, Roth RJ, Wu JJ, Jones EA, Bennett AM, Flavell RA. Dynamic regulation of pro- and anti-inflammatory cytokines by mapk phosphatase 1 (mkp-1) in innate immune responses. *Proc Natl Acad Sci U S A.* 2006;103:2274-2279

85. Thalhamer T, McGrath MA, Harnett MM. Mapks and their relevance to arthritis and inflammation. *Rheumatology (Oxford).* 2008;47:409-414

86. Medicherla S, Ma JY, Mangadu R, Jiang Y, Zhao JJ, Almirez R, Kerr I, Stebbins EG, O'Young G, Kapoun AM, Luedtke G, Chakravarty S, Dugar S, Genant HK, Protter AA. A selective p38 alpha mitogen-activated protein kinase inhibitor reverses cartilage and bone destruction in mice with collagen-induced arthritis. *J Pharmacol Exp Ther.* 2006;318:132-141

87. Badger AM, Griswold DE, Kapadia R, Blake S, Swift BA, Hoffman SJ, Stroup GB, Webb E, Rieman DJ, Gowen M, Boehm JC, Adams JL, Lee JC. Disease-modifying activity of sb 242235, a selective inhibitor of p38 mitogen-activated protein kinase, in rat adjuvant-induced arthritis. *Arthritis Rheum.* 2000;43:175-183

88. Nishikawa M, Myoui A, Tomita T, Takahi K, Nampei A, Yoshikawa H. Prevention of the onset and progression of collagen-induced arthritis in rats by the potent p38 mitogen-activated protein kinase inhibitor fr167653. *Arthritis Rheum.* 2003;48:2670-2681

89. McLay LM, Halley F, Souness JE, McKenna J, Benning V, Birrell M, Burton B, Belvisi M, Collis A, Constan A, Foster M, Hele D, Jayyosi Z, Kelley M, Maslen C, Miller G, Ouldelhkim MC, Page K, Phipps S, Pollock K, Porter B, Ratcliffe AJ, Redford EJ, Webber S, Slater B, Thybaud V, Wilsher N. The discovery of rpr 200765a, a p38 map kinase inhibitor displaying a good oral anti-arthritic efficacy. *Bioorganic & medicinal chemistry.* 2001;9:537-554

90. Mbalaviele G, Anderson G, Jones A, De Ciechi P, Settle S, Mnich S, Thiede M, Abu-Amer Y, Portanova J, Monahan J. Inhibition of p38 mitogen-activated protein kinase prevents inflammatory bone destruction. *J Pharmacol Exp Ther.* 2006;317:1044-1053

91. Han Z, Boyle DL, Chang L, Bennett B, Karin M, Yang L, Manning AM, Firestein GS. C-jun n-terminal kinase is required for metalloproteinase

expression and joint destruction in inflammatory arthritis. *Journal of Clinical Investigation*. 2001;108:73-81

92. Thirumurugan D, Cholarajan A, Raja SSS, Vijayakumar R. An introductory chapter: Secondary metabolites, secondary metabolites - sources and applications. *IntechOpen*. 2018:1-21

93. Dias DA, Urban S, Roessner U. A historical overview of natural products in drug discovery. *Metabolites*. 2012;2:303-336

94. Lee MR. Plants against malaria. Part 1: Cinchona or the peruvian bark. *J R Coll Physicians Edinb* 2002;32:189-196

95. Faurant C. From bark to weed: The history of artemisinin. *Parasite*. 2011;18:215-218

96. Achan J, Talisuna AO, Erhart A, Yeka A, Tibenderana JK, Baliraine FN, Rosenthal PJ, D'Alessandro U. Quinine, an old anti-malarial drug in a modern world: Role in the treatment of malaria. *Malar J*. 2011;10:144

97. Kim JH, Kang IS, Choi HK, Hong SS, Lee HS. A novel prepurification for paclitaxel from plant cell cultures. *Process Biochemistry*. 2002;37:679-682

98. Samuels ML, Howe CD. Vinblastine in the management of testicular cancer *Cancer*. 1970;5:1009-1017

99. G.B. S, Ptacek R, Kuzelova H, Kream RM. Endogenous morphine: Up-to-date review 2011. *Folia Biologica* 2012;58:49-56

100. Fleming A. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of b. Influenzae. *Br J Exp Pathol*. 1929;10:226-236

101. Aminov RI. A brief history of the antibiotic era: Lessons learned and challenges for the future. *Frontiers in Microbiology*. 2010;1

102. Newman DJ, Cragg GM. Natural products as source of new drugs over the last 25 years. *J Nat Prod*. 2007;70:461-477

103. Koeberle A, Werz O. Multi-target approach for natural products in inflammation. *Drug Discov Today*. 2014;19:1871-1882

104. Harvey AL. Natural products in drug discovery. *Drug Discov Today*. 2008;13:894-901

105. Ottesen EA, Campbell W. Ivermectin in human medicine. *Journal of Antimicrobial Chemotherapy*. 1994;34:195-203

106. Kahan BD, Gibbons S, Tejpal N, Stepkowski SM, Chou TC. Synergistic interactions of cyclosporine and rapamycin to inhibit immune performances of normal human peripheral blood lymphocytes in vitro. *Transplantation*. 1991;51:232-239

107. Endo A, Kuroda M, Tsujita Y. Ml-236a, ml-236b, and ml-236c, new inhibitors of cholesterogenesis produced by penicillium citrinium. *J Antibiot*. 1976;29:1346-1348

108. Seydametova E, Salihon J, Zainol N, Convey P. Production of lovastatin by penicillium spp. Soil microfungi. *International Journal of Chemical Engineering and Applications*. 2012;3:337-339

109. Hamann MT, Scheuer PJ. Kahalalide f: A bioactive depsipeptide from the sacoglossan mollusk elysia rufescens and the green alga bryopsis sp. *Journal of the American Chemical Society*. 1993;115:5825-5826

110. Cuadros R, Montejo de Garcini E, Wandosell F, Faircloth G, Fernandez-Sousa JM, Avila J. The marine compound spisulosine, an inhibitor of cell proliferation, promotes the disassembly of actin stress fibers. *Cancer letters*. 2000;152:23-29

111. Moore BM, Seaman FC, Hurley LH. Molecular recognition of DNA by ecteinascidin 743. *Recent Trends in Molecular Recognition*. 1998:81-95

112. Patridge E, Gareiss P, Kinch MS, Hoyer D. An analysis of fda-approved drugs: Natural products and their derivatives. *Drug Discov Today*. 2016;21:204-207

113. Cragg GM, Newman DJ. Biodiversity: A continuing source of novel drug leads. *Pure and Applied Chemistry*. 2005;77:7-24

114. Ganie SH, Upadhyay P, Das S, Prasad Sharma M. Authentication of medicinal plants by DNA markers. *Plant Gene*. 2015;4:83-99

115. Buriani A, Garcia-Bermejo ML, Bosisio E, Xu Q, Li H, Dong X, Simmonds MS, Carrara M, Tejedor N, Lucio-Cazana J, Hylands PJ. Omic techniques in systems biology approaches to traditional chinese medicine research: Present and future. *J Ethnopharmacol*. 2012;140:535-544

116. Koparde AA, Doijad RC, Magdum CS. *Natural products in drug discovery, pharmacognosy - medicinal plants, shagufta perveen and areej al-taweel*. IntechOpen; 2019.

117. Thomford N, Senthebane D, Rowe A, Munro D, Seele P, Maroyi A, Dzobo K. Natural products for drug discovery in the 21st century: Innovations for novel drug discovery. *International Journal of Molecular Sciences*. 2018;19:1578

118. Kiyohara H, Matsumoto T, Yamada H. Combination effects of herbs in a multi-herbal formula: Expression of juzen-taiho-to's immuno-modulatory activity on the intestinal immune system *eCAM*. 2004;1:83-91

119. Caesar LK, Cech NB. Synergy and antagonism in natural product extracts: When 1 + 1 does not equal 2. *Nat Prod Rep*. 2019;36:869-888

120. Gaulton A, Hersey A, Nowotka M, Bento AP, Chambers J, Mendez D, Mutowo P, Atkinson F, Bellis LJ, Cibrian-Uhalte E, Davies M, Dedman N, Karlsson A, Magarinos MP, Overington JP, Papadatos G, Smit I, Leach AR. The chembl database in 2017. *Nucleic Acids Res*. 2017;45:945-954

121. Zaru R, Magrane M, Orchard S, UniProt C. Challenges in the annotation of pseudoenzymes in databases: The uniprotkb approach. *FEBS J*. 2019

122. Gaulton A, Bellis LJ, Bento AP, Chambers J, Davies M, Hersey A, Light Y, McGlinchey S, Michalovich D, Al-Lazikani B, Overington JP. Chembl: A large-scale bioactivity database for drug discovery. *Nucleic Acids Res*. 2012;40:1100-1107

123. Ozdemir V. Omics 2.0: An accelerator for global science, systems medicine and responsible innovation. *OMICS*. 2015;19:579-580

124. Kornienko A, Evidente A. Chemistry, biology, and medicinal potential of narciclasine and its congeners. *Chem Rev*. 2008;108:1982-2014

125. Ceriotti G. Narciclasine: An antimitotic substance from narcissus bulbs. *Nature* 1967;213:595-596

126. Immirzi A, Fuganti C. The crystal and molecular structure of narciclasine tetra-acetate. *Journal of the Chemical Society, Chemical Communications*. 1972;4:240

127. Carrasco L, Frenso M, Vasquez D. Narciclasine: An antitumor alkaloid which blocks peptide bond formation by eukaryotic ribosomes. *FEBS Letters*. 1975;52:236-239

128. Frenso M, Carrasco L, Vascquez D. Initiation of the polypeptide chain by recruitment cell-free systems. Survey of different inhibitors of translation. *Eur J Biochem*. 1976;68:355-364

129. Van Goietsenoven G, Mathieu V, Lefranc F, Kornienko A, Evidente A, Kiss R. Narciclasine as well as other amaryllidaceae isocarbostyrils are promising

gtp-ase targeting agents against brain cancers. *Med Res Rev*. 2013;33:439-455

130. Ingrassia L, Lefranc F, Mathieu V, Darro F, Kiss R. Amaryllidaceae isocarbostyril alkaloids and their derivatives as promising antitumor agents. *Transl Oncol*. 2008;1:1-13

131. Van Goietsenoven G, Hutton J, Becker JP, Lallemand B, Robert F, Lefranc F, Pirker C, Vandenbussche G, Van Antwerpen P, Evidente A, Berger W, Prevost M, Pelletier J, Kiss R, Kinzy TG, Kornienko A, Mathieu V. Targeting of eef1a with amaryllidaceae isocarbostyrils as a strategy to combat melanomas. *FASEB J*. 2010;24:4575-4584

132. Lefranc F, Sauvage S, Van Goietsenoven G, Megalizzi V, Lamoral-Theys D, Debeir O, Spiegl-Kreinecker S, Berger W, Mathieu V, Decaestecker C, Kiss R. Narciclasine, a plant growth modulator, activates rho and stress fibers in glioblastoma cells. *Molecular Cancer Therapeutics*. 2009;8:1739-1750

133. Brautigam J, Bischoff I, Schurmann C, Buchmann G, Epah J, Fuchs S, Heiss E, Brandes RP, Furst R. Narciclasine inhibits angiogenic processes by activation of rho kinase and by downregulation of the vegf receptor 2. *J Mol Cell Cardiol*. 2019;135:97-108

134. Cao C, Huang W, Zhang N, Wu F, Xu T, Pan X, Peng C, Han B. Narciclasine induces autophagy-dependent apoptosis in triple-negative breast cancer cells by regulating the ampk-ulk1 axis. *Cell Prolif*. 2018;51:e12518

135. Dumont P, Ingrassia L, Rouzeau S, Ribaucour F, Thomas S, Roland I, Darro F, Lefranc F, Kiss R. The amaryllidaceae isocarbostyril narciclasine induces apoptosis by activation of the death receptor and/or mitochondrial pathways in cancer cells but not in normal fibroblasts. *Neoplasia*. 2007;9:766-776

136. Fürst R. Narciclasine - an amaryllidaceae alkaloid with potent antitumor and anti-inflammatory properties. *Planta Med*. 2016;82:1389-1394

137. Mikami M, Kitahara M, Kitano M, Ariki Y, Mimaki Y, Sashida Y, Yamazaki M, Yui S. Suppressive activity of lycoricidinol (narciclasine) against cytotoxicity of neutrophil-derived calprotectin, and its suppressive effect on rat asdjuvant arthritis model. *Biol Pharm Bull*. 1999;22:674-678

138. Lubahn C, Schaller JA, Shewmacker E, Wood C, Bellinger DL, Byron D, Melody N, Pettit GR, Lorton D. Preclinical efficacy of sodium narcistatin to reduce inflammation and joint destruction in rats with adjuvant-induced arthritis. *Rheumatol Int*. 2012;32:3751-3760

139. Kim J, Park Y, Chun YS, Cha JW, Kwon HC, Oh MS, Chung S, Yang HO. Effect of lycoris chejuensis and its active components on experimental models of alzheimer's disease. *J Agric Food Chem*. 2015;63:6979-6988

140. Yui M, Mikami M, Mimaki Y, Sashida Y, Yamazaki M. Inhibition effect of amaryllidaceae alkaloids, lycorine and lycoricidinol on macrophage tnf-a production. *Yakugaku Zasshi*. 2001;121:167-171

141. Yamazaki M, Kawano Y. Inhibitory effects of herbal lalkaloids on the tumor necrosis factor-a and nitric oxide production in lipopolysaccharide-stimulated raw264 macrophages. *Chem Pharm Bull*. 2011;59:388-391

142. Fuchs S, Hsieh LT, Saarberg W, Erdelmeier CA, Wichelhaus TA, Schaefer L, Koch E, Furst R. Haemanthus coccineus extract and its main bioactive component narciclasine display profound anti-inflammatory activities in vitro and in vivo. *J Cell Mol Med*. 2015;19:1021-1032

143. Weksler B, Romero IA, Couraud PO. The hcmec/d3 cell line as a model of the human blood brain barrier. *Fluids and Barriers*. 2013;10

144. Weksler BB, Subileau EA, Perriere N, Charneau P, Holloway K, Leveque M, Tricoire-Leignel H, Nicotra A, Bourdoulous S, Turowski P, Male DK, Roux F, Greenwood J, Romero IA, Couraud PO. Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *Faseb j.* 2005;19:1872-1874

145. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (thp-1). *International journal of cancer.* 1980;26:171-176

146. Schneider U, Schwenk HU, Bornkamm G. Characterization of ebv-genome negative "null" and "t" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-hodgkin lymphoma. *International journal of cancer.* 1977;19:621-626

147. Boyum A. Isolation of lymphocytes, granulocytes and macrophages. *Scand J Immunol.* 1976;5

148. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods.* 1991;139:271-279

149. Chan FK, Moriwaki K, De Rosa MJ. Detection of necrosis by release of lactate dehydrogenase activity. *Methods Mol Biol.* 2013;979:65-70

150. Biosystems A. Applied biosystems stepone™ and steponeplus™ real-time pcr systems. *Reagent Guide.* 2010

151. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. *Methods (San Diego, Calif.).* 2001;25:402-408

152. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci U S A.* 1979;79:4350-4354

153. Huang T, Long M, Huo B. Competitive binding to cuprous ions of protein and bca in the bicinchoninic acid protein assay *The Open Biomedical Engineering Journal* 2010;4:271-278

154. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage t4. *Nature.* 1970;227:680-685

155. Lewis JC, Feltus A, Ensor CM, Ramanathan S, Daunert S. Peer reviewed: Applications of reporter genes. *Analytical Chemistry.* 1998;70:579A-585A

156. Naylor LH. Reporter gene technology: The future looks bright. *Biochemical pharmacology.* 1999;58:749-757

157. Doherty NS, Poubelle P, Borgeat P, Beaver TH, Westrich GL, Schrader NL. Intraperitoneal injection of zymosan in mice induces pain, inflammation and the synthesis of peptidoleukotrienes and prostaglandin e2. *Prostaglandins.* 1985;30:796-789

158. Leite JA, Alves AK, Galvao JG, Teixeira MP, Cavalcante-Silva LH, Scavone C, Morrot A, Rumjanek VM, Rodrigues-Mascarenhas S. Ouabain modulates zymosan-induced peritonitis in mice. *Mediators Inflamm.* 2015;2015:265798

159. Laux-Biehlmann A, Boyken J, Dahllof H, Schmidt N, Zollner TM, Nagel J. Dynamic weight bearing as a non-reflexive method for the measurement of abdominal pain in mice. *Eur J Pain.* 2016;20:742-752

160. Cobos EJ, Ghasemlou N, Araldi D, Segal D, Duong K, Woolf CJ. Inflammation-induced decrease in voluntary wheel running in mice: A nonreflexive test for evaluating inflammatory pain and analgesia. *Pain.* 2012;153:876-884

161. M. B, Moos V, Schuhbauer D, Müller B. Differential expression of major histocompatibility complex class ii genes on murine macrophages associated with t cell cytokine profile and protective / suppressive effects. *Proc Natl Acad Sci U S A*. 1998;95:6936

162. Cella M, A. E, Pinet V, Pieters J, Lanzavecchia A. Inflammatory stimuli induce accumulation of mhc class ii complexes on dendritic cells. *Nature*. 1997;388:782-787

163. Breslin WL, Strohacker K, Carpenter KC, Haviland DL, McFarlin BK. Mouse blood monocytes: Standardizing their identification and analysis using cd115. *J Immunol Methods*. 2013;390:1-8

164. Hickey MJ. Has ly6g finally found a job? *Blood*. 2012;120:1352-1353

165. Rose S, Misharin A, Perlman H. A novel ly6c/ly6g-based strategy to analyze the mouse splenic myeloid compartment. *Cytometry A*. 2012;81:343-350

166. Morgan E, Varro R, Sepulveda H, Ember JA, Apgar J, Wilson J, Lowe L, Chen R, Shivraj L, Agadir A, Campos R, Ernst D, Gaur A. Cytometric bead array: A multiplexed assay platform with applications in various areas of biology. *Clin Immunol*. 2004;110:252-266

167. Baez S. An open cremaster muscle preparation for the study of blood vessels by in vivo microscopy. *Microvasc Res*. 1973;5:384-394

168. Bakhle YS, Botting RM. Cyclooxygenase-2 and its regulation in inflammation. *Mediators of Inflammation*. 1996;5

169. Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol*. 2011;31:986-1000

170. Knaus UG. Rho gtpase signaling in inflammation and transformation. *Immunologic Research*. 2000;21:103-109

171. Sit ST, Manser E. Rho gtpases and their role in organizing the actin cytoskeleton. *Journal of Cell Science*. 2011;124

172. Tong L, Tergaonkar V. Rho protein gtpases and their interactions with nfkappab: Crossroads of inflammation and matrix biology. *Biosci Rep*. 2014;34:283-295

173. Lemon B, Tjian R. Orchestrated response: A symphony of transcription factors for gene control. *Genes & Development* 2000;14:2551-2569

174. Bosman-den Boer MM, Van Wetten ML, Pruimboom L. Chronic inflammatory diseases are stimulated by current lifestyle: How diet, stress levels and medication prevent our body from recovering. *Nutrition & Metabolism*. 2012;9:1-14

175. Schett G, Neurath MF. Resolution of chronic inflammatory disease: Universal and tissue-specific concepts. *Nat Commun*. 2018;9:3261

176. Danese S, Panes J. Development of drugs to target interactions between leukocytes and endothelial cells and treatment algorithms for inflammatory bowel diseases. *Gastroenterology*. 2014;147:981-989

177. Selewski DT, Shah GV, Segal BM, Rajdev PA, Mukherji SK. Natalizumab (tysabri). *AJNR Am J Neuroradiol*. 2010;31:1588-1590

178. Rudick RA, Stuart WH, Calabresi PA, Confavreux C, Galetta SL, Radue EW, Lublin FD, Weinstock-Guttman B, Wynn DR, Lynn F, Panzara MA, Sandrock AW, Investigators S. Natalizumab plus interferon beta-1a for relapsing multiple sclerosis. *N Engl J Med*. 2006;354:911-923

179. Soler D, Chapman T, Yang LL, Wyant T, Egan R, Fedyk ER. The binding specificity and selective antagonism of vedolizumab, an anti-alpha4beta7 integrin therapeutic antibody in development for inflammatory bowel diseases. *J Pharmacol Exp Ther*. 2009;330:864-875

180. Gordon FH, Lai CW, Hamilton MI, Allison MC, Srivastava ED, Fouweather MG, Donoghue S, Greenlees C, Subhani J, Amlot PL, Pounder RE. A randomized placebo-controlled trial of a humanized monoclonal antibody to alpha4 integrin in active crohn's disease. *Gastroenterology*. 2001;121:268-274

181. Raine T. Vedolizumab for inflammatory bowel disease: Changing the game, or more of the same? *United European Gastroenterol J*. 2014;2:333-344

182. Kingsley MK, Bhat BV, Badhe BA, Dhas BB, Parija SC. Narciclasine improves outcome in sepsis among neonatal rats via inhibition of calprotectin and alleviating inflammatory responses. *Sci Rep*. 2020;10:2947

183. Sikandar S, Dickenson AH. Visceral pain: The ins and outs, the ups and downs. *Curr Opin Support Palliat Care*. 2012;6:17-26

184. Sengupta JN. Visceral pain: The neurophysiological mechanism. *Handb Exp Pharmacol*. 2009;194:31-74

185. Lee YC. Effect and treatment of chronic pain in inflammatory arthritis. *Curr Rheumatol Rep*. 2013;15:300

186. Courvoisier DS, Agoritsas T, Glauser J, Michaud K, Wolfe F, Cantoni E, Perneger TV, Finckh A, Swiss Clinical Quality Management Program for Rheumatoid A, National Data Bank for Rheumatic D. Pain as an important predictor of psychosocial health in patients with rheumatoid arthritis. *Arthritis Care Res (Hoboken)*. 2012;64:190-196

187. Haverman L, Grootenhuis MA, van den Berg JM, van Veenendaal M, Dolman KM, Swart JF, Kuijpers TW, van Rossum MA. Predictors of health-related quality of life in children and adolescents with juvenile idiopathic arthritis: Results from a web-based survey. *Arthritis Care Res (Hoboken)*. 2012;64:694-703

188. Gupta SC, Sundaram C, Reuter S, Aggarwal BB. Inhibiting nf-kappab activation by small molecules as a therapeutic strategy. *Biochim Biophys Acta*. 2010;1799:775-787

189. Jobin C, Bradham CA, Russo MP, Juma B, Narula AS, Brenner DA, Sartor RB. Curcumin blocks cytokine-mediated nf-кb activation and proinflammatory gene expression by inhibiting inhibitory factor iкb kinase activity. *J Immunol*. 1999;163:3474-3483

190. Epinat JC, Golmore TD. Diverse agents act at multiple levels to inhibit the rel/nf-кb signal transduction pathway. *Oncogene*. 1999;18:6896-6909

191. Morgan MJ, Liu ZG. Crosstalk of reactive oxygen species and nf-kappab signaling. *Cell Res*. 2011;21:103-115

192. Gloire G, Legrand-Poels S, Piette J. Nf-kappab activation by reactive oxygen species: Fifteen years later. *Biochemical pharmacology*. 2006;72:1493-1505

193. Holmes-McNary M, Baldwin Jr AS. Chemopreventive properties of trans-resveratrol are associated with inhibition of activation of the iкb kinase. *Cancer Research*. 2000;60:3477-3483

194. Tsai SH, Lin-Schiau SY, Lin JK. Suppression of nitric oxide synthase and the down-regulation of the activation of nf-кb in macrophages by resveratrol. *British Journal of Pharmacology*. 1999;126:673-680

195. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma I. Suppression of tnf-alpha-induced apoptosis by nf-kappab. *Science*. 1996;274:787-789

196. Wang CY, Mayo MW, Baldwin AS. Tnf- and cancer therapy-induced apoptosis: Potentiation by inhibition of nf-kappab. *Science*. 1996;274:784-787

197. Baliga BS, Pronczuk AW, Munro HN. Mechanism of cycloheximide inhibition of protein synthesis in a cell-free system prepared from rat liver *The Journal of Biological Chemistry*. 1969;224:4480-4489

198. Obrig TG, Culp WJ, McKeehan WL, Hardesty B. The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes *J Biol Chem*. 1971;246:174-181

199. Ciechanover A. Intracellular protein degradation: From a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. *Cell Death Differ*. 2005;12:1178-1190

200. Huber LA, Teis D. Lysosomal signaling in control of degradation pathways. *Curr Opin Cell Biol*. 2016;39:8-14

201. Kisselev AF, Goldberg AL. Proteasome inhibitors: From research tools to drug candidates. *Chemistry & Biology*. 2001;8:739-758

202. Popa C, Netea MG, Van Riel PL, Van der Meer JW, Stalenhoef AF. The role of tnf-alpha in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk. *J Lipid Res*. 2007;48:751-762

203. Sedger LM, McDermott MF. Tnf and tnf-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future. *Cytokine Growth Factor Rev*. 2014;25:453-472

204. Willoughby DA, Moore AR, Colville-Nash PR. Cox-1, cox-2, and cox-3 and the future treatment of chronic inflammatory disease. *The Lancet*. 2000;355:646-648

205. Seibert K, Masferrer JL. Role of inducible cyclooxygenase (cox-2) in inflammation. *Receptor*. 1994;4:17-23

206. Williams CS, Mann M, DuBois RN. The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene*. 1999;18:7908-7916

207. Chan MM, Moore AR. Resolution of inflammation in murine autoimmune arthritis is disrupted by cyclooxygenase-2 inhibition and restored by prostaglandin e2-mediated lipoxin a4 production. *J Immunol*. 2010;184:6418-6426

208. Mattia C, Coluzzi F. Cox-2 inhibitors: Pharmacological data and adverse events. *Minerva Anestesiol*. 2005;71:461-470

209. Ray WA, Stein CM, Daugherty JR, Hall K, Arbogast PG, Griffin MR. Cox-2 selective non-steroidal anti-inflammatory drugs and risk of serious coronary heart disease. *The Lancet*. 2002;360:1071-1073

210. Juni P, Nartey L, Reichenbach S, Sterchi R, Dieppe PA, Egger M. Risk of cardiovascular events and rofecoxib: Cumulative meta-analysis. *Lancet (London, England)*. 2004;364:2021-2029

211. Segain JP, Raingeard de la Blétière D, Sauzeau V, Bourreille A, Hilaret G, Cario-Toumaniantz C, Pacaud P, Galmiche JP, Loirand G. Rho kinase blockade prevents inflammation via nuclear factor κb inhibition: Evidence in crohn's disease and experimental colitis. *Gastroenterology*. 2003;124:1180-1187

212. He Y, Xu H, Liang L, Zhan Z, Yang X, Yu X, Ye Y, Sun L. Antiinflammatory effect of rho kinase blockade via inhibition of nf-kappab activation in rheumatoid arthritis. *Arthritis Rheum*. 2008;58:3366-3376

213. Stark A, Schwenk R, Wack G, Zuchtriegel G, Hatemler MG, Brautigam J, Schmidtko A, Reichel CA, Bischoff I, Furst R. Narciclasine exerts anti-inflammatory actions by blocking leukocyte-endothelial cell interactions and down-regulation of the endothelial tnf receptor 1. *FASEB J*. 2019;33:8771-8781

214. Shen CY, Xu XL, Yang LJ, Jiang JG. Identification of narciclasine from lycoris radiata (l'her.) herb. And its inhibitory effect on lps-induced inflammatory responses in macrophages. *Food Chem Toxicol.* 2019;125:605-613

215. Fraser CC. Exploring the positive and negative consequences of nf kappa b inhibition for the treatment of human disease. *Cell Cycle.* 2006;5:1160-1163

216. Kelly C, Hamilton J. What kills patients with rheumatoid arthritis? *Rheumatology (Oxford).* 2007;46:183-184

217. Sokka T, Kautiainen H, Pincus T, Verstappen SM, Aggarwal A, Alten R, Andersone D, Badsha H, Baecklund E, Belmonte M, Craig-Muller J, da Mota LM, Dimic A, Fathi NA, Ferraccioli G, Fukuda W, Geher P, Gogus F, Hajjaj-Hassouni N, Hamoud H, Haugeberg G, Henrohn D, Horslev-Petersen K, Ionescu R, Karateew D, Kuuse R, Laurindo IM, Lazovskis J, Luukkainen R, Mofti A, Murphy E, Nakajima A, Oyoo O, Pandya SC, Pohl C, Predeteanu D, Rexhepi M, Rexhepi S, Sharma B, Shono E, Sibilia J, Sierakowski S, Skopouli FN, Stropuviene S, Toloza S, Valter I, Woolf A, Yamanaka H, Quest RA. Work disability remains a major problem in rheumatoid arthritis in the 2000s: Data from 32 countries in the quest-ra study. *Arthritis Res Ther.* 2010;12:R42

6.1 Internet links

https://smart.servier.com/

7 APPENDIX

7.1 Declaration

7.1.1 Data obtained in cooperation

All data in this PhD thesis was generated by me under the supervision of Prof. Dr. Robert Fürst, unless stated otherwise by reference, acknowledgement or this declaration. Material for figures listed below was obtained as the result of collaboration with colleagues.

Figure 8: Narciclasine demonstrates anti-inflammatory effects in *in vivo* zymosan-induced murine peritonitis model. *In vivo* experiments and dynamic weight bearing data analysis were performed by Gesine Wack (Institute of Pharmacology and Clinical Pharmacy, Goethe University, Frankfurt/Main, Germany). Identification of monocytes and neutrophils in the peritoneal lavage and concentration of TNF, IL-1β and IL-6 were performed by me, as well as preparation of the complete figure.

Figure 9: Narciclasine reduces rolling, adhesion and transmigration in the *in vivo* cremaster muscle model. *In vivo* experiments and data analysis were performed by Gabriele Zuchtriegel (Department of Otorhinolaryngology, Head and Neck Surgery and Walter Brendel Center of Experimental Medicine, University of Munich, Munich, Germany). Figure preparation was done by me.

Figure 12: Narciclasine inhibits the THP-1 adhesion to HUVECs monolayer under flow conditions. Experiments and data analysis were performed by Dr. Rebecca Ingelfinger and Dr. Iris Bischoff-Kont (Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany). Figure preparation was done by me.

Figure 13: Narciclasine reduces the TNF-triggered adhesion of leukocytes to an endothelial cell monolayer. Cell adhesion assay with HUVEC and PMNL and data analysis were performed by Melissa Krishnathas from the Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany. Figure preparation was done by me.

Figure 14: Narciclasine reduces adhesion of THP-1 cells and Jurkat cells when added to HUVEC monolayer before and after TNF activation. Experiments and data analysis were performed by Dr. Rebecca Ingelfinger **(A)** and

Dr. Iris Bischoff-Kont **(B)** from the Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany. Figure preparation was done by me.

Figure 15: Narciclasine reduces transmigration of leukocytes through the endothelial cell layer. Data were obtained and analyzed by Dr. Rebecca Ingelfinger from the Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany. Figure preparation was done by me.

Figure 24: Narciclasine inhibits the translocation of the p65 subunit of the NF-κB complex into the nucleus. Experiments and data analysis for the part **(B)** of this figure were done by Dr. Rebecca Ingelfinger from the Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany. Figure preparation was done by me.

Figure 27: Narciclasine inhibits the TNF-induced phosphorylation of TAK-1. One out of three western blots were performed by Dr. Iris Bischoff-Kont from the Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany. Data quantification and figure preparation was done by me.

Figure 28: Narciclasine blocks TNF receptor 1 (TNFR1) expression independent of the action of proteasomal inhibitor MG-132. Experiments and data analysis were performed by Dr. Rebecca Ingelfinger and Dr. Iris Bischoff-Kont (Institute of Pharmaceutical Biology, Goethe University, Frankfurt/Main, Germany). Figure preparation was done by me.

7.1.2 Previously published data

Some of the figures presented in this work were previously published in the following paper:

Anna Stark, Rebecca Schwenk, Gesine Wack, Gabriele Zuchtriegel, Melissa G. Hatemler, Jacqueline Bräutigam, Achim Schmidtko, Christoph A. Reichel, Iris Bischoff, Robert Fürst. Narciclasine exerts anti-inflammatory actions by blocking leukocyte-endothelial cell interactions and down-regulation of the endothelial TNF receptor 1. FASEB J. 2019 Aug;33(8):8771-8781.

The following figures have been published in the above-mentioned paper prior to the submission of this PhD thesis:

Figure 8: Narciclasine demonstrates anti-inflammatory effects in *in vivo* zymosan-induced murine peritonitis model.

Figure 9: Narciclasine reduces rolling, adhesion and transmigration in the *in vivo* cremaster muscle model.

Figure 10: Narciclasine does not cause apoptosis of HUVEC up to 1 µM concentration.

Figure 11: Narciclasine has no cytotoxic effects on HUVEC up to 1 µM concentration.

Figure 12: Narciclasine inhibits the THP-1 adhesion to HUVECs monolayer under flow conditions. Part **(B)** was published.

Figure 13: Narciclasine reduces the adhesion of leukocytes to an endothelial cell monolayer.

Figure 14: Narciclasine reduces adhesion of THP-1 cells and Jurkat cells when added to HUVEC monolayer before and after TNF activation.

Figure 15: Narciclasine reduces transmigration of leukocytes through the endothelial cell layer.

Figure 16: Narciclasine strongly reduces VCAM-1 mRNA, total protein and surface protein expression in endothelial cells.

Figure 17: Narciclasine strongly reduces ICAM-1 mRNA, total protein and surface protein expression in endothelial cells.

Figure 18: Narciclasine upregulates E-selectin mRNA expression but downregulates its total protein and surface protein expression in HUVECs.

Figure 19: Narciclasine downregulates CX3CL1 mRNA and total protein expression in HUVECs.

Figure 20: Narciclasine inhibits the activity of the NF-κB promoter.

Figure 22: Rho and ROCK are not of importance for narciclasine's ability to downregulate ICAM-1 expression and Jurkat adhesion to HUVECs in response to TNF treatment. Part **(A)** was published.

Figure 23: Narciclasine inhibits the activity of a TNF-induced artificial NF-κB promoter.

Figure 25: Narciclasine inhibits TNF-induced phosphorylation of IκBα and impairs IκBα proteasomal degradation.

Figure 26: Narciclasine inhibits TNF-induced phosphorylation of IKKβ.

Figure 27: Narciclasine inhibits TNF-induced phosphorylation of TAK-1.

Figure 28: Narciclasine lowers TNF receptor 1 (TNFR1) levels. Part **(B)** was published.

Figure 29: Narciclasine lowers TNF receptor 1 (TNFR1) levels independent of the proteasome.

Figure 30: Narciclasine blocks TNF receptor 1 (TNFR1) expression, but does not reduce IκBα, IKKβ and TAK-1 total protein expression.

Figure 31: Narciclasine does not have an influence on IL-1 receptor 1 (IL1R1) expression.

7.2 Supplement
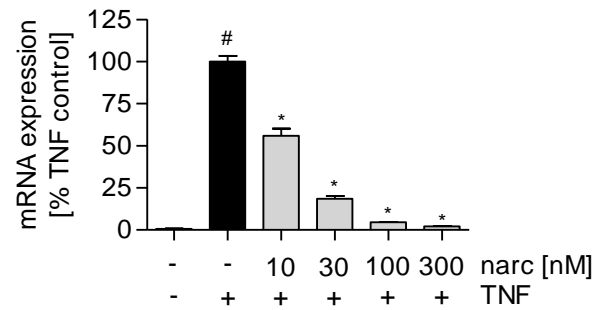
Supplement Figure I: Narciclasine downregulates ACKR-1 mRNA expression in HUVECs.
HUVEC were pretreated with narciclasine (10, 30, 100, 300 nM) for 30 min before the activation with TNF (10 ng/ml) for 12 h. For mRNA expression analysis qPCR was performed. Data are expressed as mean ± SEM; n=3; *p ≤ 0.05 *vs.* TNF control, #p ≤ 0.05 *vs.* negative control.

7.3 Abbreviations

Table 19: List of Abbreviations

Abbreviation	Full name
ACKR-1	Atypical chemokine receptor-1
AP-1	Activator protein-1
APS	Ammonium persulfate
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
Blotto-T	Powdered milk in tris buffered saline plus Tween® 20
BSA	Bovine serum albumin
CBA	Cytometric Bead Array
cDNA	Complementary deoxyribonucleic acid
CD99	Cluster of differentiation 99
COX2	Cyclooxygenase-2
CHX	Cycloheximide
CX3CL1	Fractalkine
CX3CR1	Fractalkine receptor
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
DMSO	Dimethyl sulfoxide
dsDNA	Double-stranded deoxyribonucleic acid
DTT	1,4-Dithiothreitol
DUSP-1	Dual specificity phosphotase-1
EC	Endothelial cell
ECGM	Endothelial Cell Growth Medium
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
E-selectin	Endothelial-leukocyte adhesion molecule 1
ESL-1	E-selectin ligand-1
FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
FACS	Fluorescence-activated cell sorting
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H_2O	Water
H_2O_2	Hydrogen peroxide
HBSS	Hanks' balanced salt solution
HCE	*Haemanthus coccineus* extract

Table 19: List of Abbreviations (continued)

Abbreviation	Full name
hCMEC/D3	Human cerebral microvascular endothelial cells
HFS	Hypotonic fluorochrome solution
HRP	Horseradish peroxidase
HTAB	Hexadecyltrimethylammoniumbromide
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IκBα	Nuclear factor-κB inhibitor alpha
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL	Interleukin
IL1R-1	Interleukin 1 receptor-1
i.p.	Intraperitoneal injection
JNK	c-Jun N-terminal kinase
LAR II	Luciferase Assay Reagent II
LDH	Lactate dehydrogenase
LFA-1	Leukocyte function associated antigen-1
LPS	Lipopolysaccharide
Mac-1	Macrophage-1 antigen
MAPK	Mitogen-activated protein kinase
MECGM	Microvascular endothelial cell growth medium
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
Narc	Narciclasine
NO	Nitric oxide
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor-κB
PAF	Platelet activation factor
PAF-R	Platelet activation factor receptor
PB	Phosphate buffer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBSA	Phosphate-buffered saline with BSA
PECAM-1	Platelet/endothelial cell adhesion molecule-1
PL	Primary lymphocytes
PI	Propidium Iodide
PMNL	Polymorphonuclear leukocyte
PSGL-1	P-selectin glycoprotein ligand-1
PVDF	Polyvinylidene difluoride

Table 19: List of Abbreviations (continued)

Abbreviation	Full name
qRT-PCR	Quantitative real-time reverse transcription polymerase chain reaction
RNase	Ribonuclease
RNA	Ribonucleic acid
RhoA	Ras homologous (Rho) protein family member A
ROCK	Rho-associated protein kinase (
RT	Room temperature
SDF-1	Stromal cell-derived factor-1
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
s-Le$_x$	Sialyl-Lewis$_x$ carbohydrate antigen
Stsp	Staurosporine
TRAF-2	TNFR-associated factor 2
TBS	Tris-buffered seline
TBS-T	Tris-buffered saline with Tween®20
T/E	Trypsin/ethylenediaminetetraacetic acid
TEMED	Tetramethylethylendiamin
TNF-α	Tumor necrosis factor-α
TNFR1	Tumor necrosis factor-α receptor 1
VCAM-1	Vascular cell adhesion protein-1
VE-cadherin	Vascular endothelial cadherin
VLA-4	Very late antigen-4
Zym	Zymosane

7.4 List of figures

7.4.1   List of supplemental figures

7.5 List of tables

7.6 Publications

7.6.1 Scientific papers

<u>Anna Stark</u>, Rebecca Schwenk, Gesine Wack, Gabriele Zuchtriegel, Melissa G. Hatemler, Jacqueline Bräutigam, Achim Schmidtko, Christoph A. Reichel, Iris Bischoff, Robert Fürst. Narciclasine exerts anti-inflammatory actions by blocking leukocyte-endothelial cell interactions and down-regulation of the endothelial TNF receptor 1. FASEB J. 2019 Aug;33(8):8771-8781.

Daniel K. Glatzel, Andreas Koeberle, Helmut Pein, Konstantin Loeser, <u>Anna Stark</u>, Nelli Keksel, Oliver Werz, Rolf Müller, Iris Bischoff, Robert Fürst. Acetyl-CoA carboxylase 1 regulates endothelial cell migration by shifting the phospholipid composition. J Lipid Res. 2018 Feb;59(2):298-311. doi: 10.1194/jlr.M080101. Epub 2017 Dec 5.

7.6.2 Conference abstracts and poster presentations

<u>Anna Stark</u>, Gabriele Zuchtriegel, Christoph A. Reichel, Iris Bischoff, Robert Fürst. The anti-inflammatory action of the Amaryllidaceae alkaloid narciclasine is based on the inhibition of leukocyte-endothelial cell interaction. 65th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research; Basel, Switzerland, 3-7 September 2017 (Poster Presentation). Abstract published in Planta Medica International Open; 2017; 4 (S 01); p. 43.

<u>Anna Waclawek</u>, Gabriele Zuchtriegel, Christoph A. Reichel, Iris Bischoff, Robert Fürst. The Amaryllidaceae alkaloid narciclasine exerts profound anti-inflammatory actions *in vitro* and *in vivo* by blocking leukocyte-endothelial cell interaction. 9th Joint Natural Product Conference; Copenhagen, Denmark, 24-27 July 2016 (Poster Presentation). Abstract published in Planta Medica; 2016; 82(S 01); p. 937.

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First of all, I would like to thank Prof. Dr. Robert Fürst for giving me an opportunity to be a part of his working group and to perform my PhD Thesis under his supervision. It was a great scientific journey that defined me as a researcher. I am grateful for the chance to attend scientific conferences during my time as a PhD student. I also would like to thank him for an "open door policy". With this I always knew I could reach out for a scientific advice at any time and this helped to reach my project goal at a faster pace. I am very thankful to Prof. Dr. Fürst for his guidance in the preparation of this thesis and for being patient with me during this process. I am truly grateful for all experiences I encountered during my time as a PhD student.

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