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Investigation of uptake and distribution of drugs via the lymphatic system

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Meinen Großeltern Ursula und Heinrich, meinen Eltern Susanne und Dirk und meiner Schwester Sina-Louisa.

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I. LIST OF ABBREVIATIONS

°C	Degree Celsius
µL	Microliter
µm	Micrometer
2D	2-dimensional
3R	Replace, Reduce, Refine
AAALAC	Association for assessment and accreditation of laboratory animal care
ADA	Antidrug antibody
ADME	Absorption, distribution, metabolism and excretion
ASD	Amorphous solid dispersions
ANOVA	Analysis of variance
API	Active pharmaceutical ingredients
AUC	Area under the curve
ARRIVE	Animal Research: Reporting of In Vivo Experiments
Batch ID	Batch identification number
Bcl	B-cell lymphoma
BCR	B-cell receptor complex
Caco-2	Colorectal adenocarcinoma cell line 2
CD	Cluster of differentiation
C_{max}	Maximum concentration (plasma, serum or tissue)
CNS	Central nervous system
CRA	Cannabinoid receptor agonist
CSF	Cerebrospinal fluid
Da	Dalton
DDT	Dichlorodiphenyltrichloroethane
dISF	Dermal interstitial fluid
DMEM	Gibco Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMPK	Drug Metabolism and Pharmacokinetics
DMSO	Dimethyl sulfoxide
dOMF	Dermal open flow microperfusion
e.g.	For example
EDTA	Ethylenediaminetetraacetic acid
etc.	*et cetera*
et al.	*et alii*
EU	European Union
FACS	Fluorescence activated cell sorting
FELASA	Federation for Laboratory Animal Science Associations
g	Gram
G	g-Force
GIS	Global internal standard
HEVs	High endothelial veins
hr	Hour
ID	Inner diameter

Ig	Immunoglobulin
ISF	Interstitial fluid
i.e.	*id est*
i.v.	Intravenous
kDa	Kilodalton
kg	Kilogram
LAT	Linker for activation of t-cells
log P	Logarithm of the octanol-water partition coefficient
LYVE-	Lymphatic vessel endothelial hyaluronan receptor
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
mg	Milligram
mL	Milliliter
mm	Millimeter
mmHg	Millimeter mercury
min	Minutes
ng	Nanogram
OD	Outer diameter
OECD	Organization for Economic Cooperation and Development
OFM	Open flow microperfusion
PEG400	Polyethylene glycol 400
pH	*pondus hydrogenii*
pK_a	Acid dissociation constant, K_a,
PREPARE	Planning Research and Experimental Procedures on Animals: Recommendations for Excellence
PROX	Prospero homeobox
rpm	Rounds per minute
s.c.	Subcutaneous
SYRCLE	Systematic Review Center for Laboratory animal Experimentation
TCR	T-cell receptor
T_{max}	Time of maximal concentration (plasma, serum or tissue)
V.	Vena
VLDL	Very low-density lipoproteins
VEGFR	Vascular endothelial growth factor

II. INTRODUCTION

The efficacy of medicinal products depends on their availability at the intended biological target site which for systemic therapeutics can be described by the presence of the free drug in the blood stream. The fraction of the administered drug that reaches systemic circulation after intake via the different routes is known as bioavailability and in case of oral drugs, it largely depends on intestinal absorption and hepatic first-pass metabolism (Toutain & Bousquet-mélou, 2004). Medical indications requiring high drug doses or the treatment of comorbid patients with numerous medications, especially in older age, are often associated with the intake of a number of daily oral dosages, which represents a burden for the respective individual (Hajjar *et al.*, 2007). As a result of high pill burden, patients are also more likely to not take their treatments as prescribed (Farrell *et al.*, 2013). To avoid a high pill burden it is beneficial to increase the oral bioavailability. Thus, understanding of oral bioavailability is a key step in drug development of both, investigational and already existing drugs. In addition to the blood circulation, it is recognized that transport via the lymphatic system represents another important path in the overall drug distribution, but detailed mechanisms are not well understood. Physicochemical properties such as high lipophilicity (log P > 5) and high molecular weight (> 500 Dalton) are limiting factors for oral bioavailability (Lipinski *et al.*, 1997). Lipid based formulations are believed to enhance the lymphatic absorption of such lipophilic drugs (Trevaskis *et al.*, 2015b). In order to increase the success of oral therapy and to make treatment more convenient and tolerable for patients, the highest possible active substance concentration per tablet or capsule should be achieved by appropriate formulation. In addition, the lymphatic system plays a major role as it is the primary route for metastases of solid tumors and the transport pathway for T- and B-lymphocytes and also provides the avoidance of the first pass effect following oral administration (El-Kattan & Varma, 2012). Therefore, understanding the intestinal lymphatic pathway could improve the development also of immunomodulatory and antineoplastic drugs (Charman, 2000).

Another common route of administration, especially for large molecules requiring the parenteral route, is subcutaneous administration. The disadvantage of subcutaneously administered drugs is that the dose volume which can be administered per injection site is limited. Also in these cases, improved bioavailability and reduced volume could improve patient compliance and open up new indication areas for previously approved drugs.

From the literature it is known that medications are transported from the application site directly into the blood stream, but also indirectly via the lymphatic system into the circulation (Wu *et al.*, 2011). The latter route via the lymph plays a major role in both, very fat-soluble substances after oral administration (Wu *et al.*, 2011) and drugs that are administered subcutaneously (Porter & Charman, 2001). Because of the flow rate being approximately 100-500 times lower than in the blood, smaller shear stresses of the lymph vessels and the presence and function of lymph nodes, this form of absorption pathway provides advantages

(Swartz, 2001). One described advantage is less frequent administrations compared to intravenous (i.v.) dosing, which is explained by a delay in reaching maximum plasma concentration and by a prolonged terminal half-life (McLennan *et al.*, 2005).

Physicochemical properties of a drug as well as its dosage form and formulation are important factors influencing drug absorption and bioavailability. Research animal models are crucial in preclinical drug development to investigate the role of these factors on the absorption, distribution, metabolism and excretion (ADME) profile of molecules in order to identify the optimal drug and formulation properties for human use. The investigation of the lymphatic compartment in this interplay requires the analysis of lymphatic components as well as the drug itself in the lymphatic fluid.

Several techniques for the collection of lymphatic fluid in animal models have been described (Bollman *et al.*, 1948; Gallo-Torres & Miller, 1969; Girardet, 1975; Noguchi *et al.*, 1985; Hauss *et al.*, 1998; Ionac, 2003; Boyd *et al.*, 2004; Li *et al.*, 2011; Trevaskis *et al.*, 2015b) and were assessed for their suitability to achieve the aim of the thesis.

This thesis is intended to investigate the lymphatic uptake and distribution of medicinal products for human use, the influence of formulation modification on the lymphatic uptake and potential modifications induced by drugs within the lymphatic system. Insights from these investigations might lead to improvement of their respective formulation development to increase bioavailability. The project consisted of two phases. First, a surgical method was established to cannulate the lymphatic system in rats. In the second phase, the uptake, modification and distribution of drugs in the lymphatic system and their release into the circulation was explored using tool compounds. Orally administered, fat-soluble small molecules and subcutaneously administered large molecules were both investigated, as lymphatic absorption and transport is most relevant for these kinds of drugs and its delivery. This approach allowed to determine factors that may positively influence the bioavailability of these molecules. In addition, for large molecules it could be shown how these drugs interact with cells on their way from the subcutis through the lymph nodes and vessels into the circulation and how this interaction can influence the immune system. The pharmacokinetics and pharmacodynamics of drugs directly in skin, fatty and brain tissue can be investigated by open flow Microperfusion (OFM) (Pieber *et al.*, 2013). Dermal open flow Microperfusion (dOMF) is particularly suitable for the analysis of large, lipophilic substances directly from the dermal interstitial fluid (dISF) (Dragatin *et al.*, 2016). The advantage of the cannulation of the lymphatic system is that it enables the investigation of the dynamics of the transition from the subcutis via the lymph into the blood stream. Furthermore, it can provide important additional information such as interaction of large molecules with the immune system in the local lymph nodes.

III. LITERATURE

1 The lymphatic system

1.1 Historical background

2400 years ago, the lymphatic system was discovered by the ancient Greeks. Hippocrates distinguished between “white”, “red” and “blue” vessels (Lulay, 2017). In the 17th century, the vascular blood circulation was described by William Harvey and the lymphatic anatomy started to enjoy significant attention (Swartz, 2001). On July 23rd 1622, an Italian anatomist, Gaspare Aselli, rediscovered the ’lacteal´ vessels during a vivisection of a ‘well fed dog’ and systematically studied the significance of these vascular structures (Natale *et al.*, 2017). He was able to figure out that these white vessels have motor activity, absorb, and transport the products of intestinal digestion, a function previously attributed to the veins (Elke, 1995). In 1627, he registered four woodcuts in his scripture ‘De lactibus’, the first anatomical color pictures of the lymphatic system. Some of his theories showed anatomical mistakes, e.g. the mesenteric lymph vessels lead into the liver which was later corrected by other investigators (Natale *et al.*, 2017). Some years later, in 1648, Jean Pecquet, an anatomist from France, observed the *Cisterna Chyli* and the *Thoracic Duct* (Chikly, 1997). Initially, he wanted to examine the contractions of a canine heart. After removing the still beating heart, he noticed the white fluid draining from the severed *V. cava cranialis*. It dawned upon him that this white substance, coming from the cisterna chyli, is the lymph fluid from all parts of the body (Loukas *et al.*, 2011). He described the transport of the chyle, another naming for the lymph, to the subclavian veins and disproved the assumption that lymph vessels empty into the liver.

This was the first time someone described the direct linkage between the lymphatic system and systemic circulation (Suy *et al.*, 2016). Even though the anatomy of the lymphatic system was already described in detail in the early 19th century, it still remains one of the most poorly understood systems of the body (Swartz, 2001).

1.2 Anatomy

Blind-ending lymph capillaries are distributed through the whole body in the parenchymal spaces, within the subcutaneous tissue and mucous membranes. Exceptions are the central nervous system (CNS) and avascular tissues, such as the optic cornea, lens and cartilage (Hawley *et al.*, 1995). Lymphatic capillaries are direct neighbors to blood capillaries and collect the lymph from the interstitial fluid and from plasma exudate (Hansen *et al.*, 2015).

The lymph capillaries, with a highly variable diameter of 10-60 µm, are built of a single layer of endothelial cells, which are highly attenuated and consists of a discontinuous basement membrane (McLennan *et al.*, 2005). The endothelial cells forming the lymph vessels are loosely adherent and overlap to form ‘cleft-like’ intercellular junctions (Hansen *et al.*, 2015). These junctions form uninterrupted channels beginning in the interstitium and leading into

the capillary lumen, which open and close in response to changes in interstitial volume and pressure (**Figure 1**). This structure ensures that small particles and macromolecules are absorbed from the interstitial space (Kagan, 2014). In contrast to the lymph capillaries, blood capillaries are characterized by the presence of tight junctions between the endothelial cells and the presence of a continuous basement membrane. This structural difference prevents the free exchange of macromolecules and particulates (McLennan *et al.*, 2005).

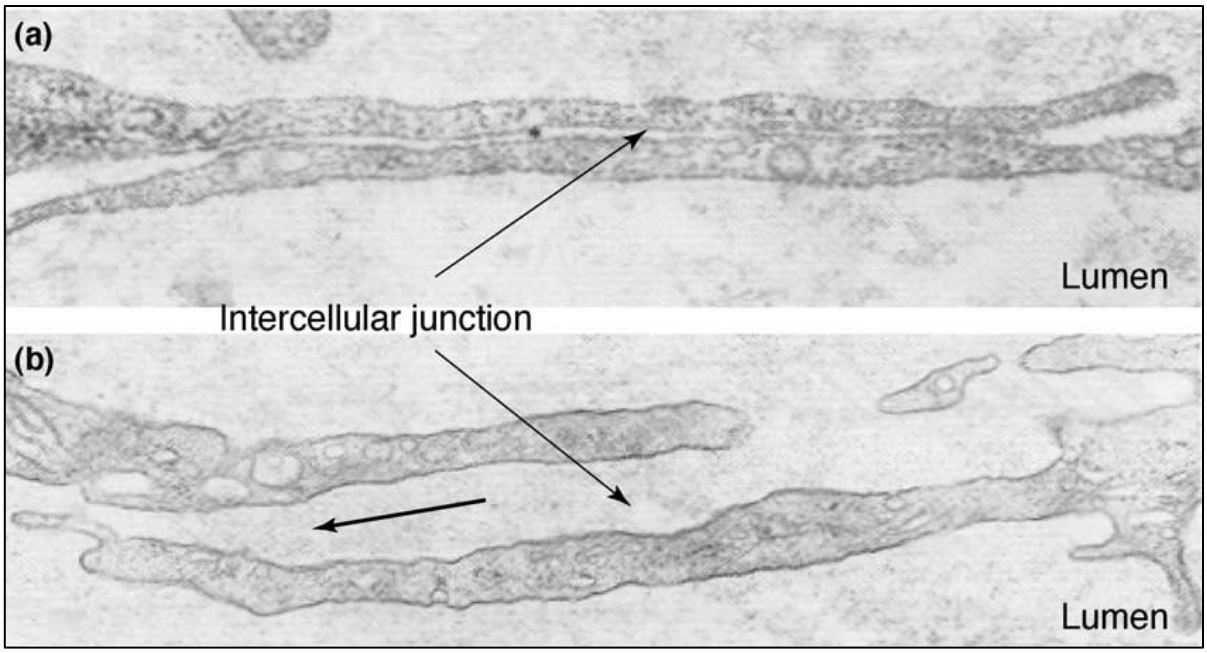


Figure 1: *Microscopic view of the lymphatic capillary a) closed (39-fold magnification) and b) opened uninterrupted channels (28-fold magnification). (Reprinted from Drug Discovery Today: Technologies, 2, 89-96., McLennan, D.N., Porter, C.J. & Charman, S.A. (2005), Subcutaneous drug delivery and the role of the lymphatics, with permission from Elsevier and the Rockefeller University Press (Leak, 1971)).*

The lymph capillaries merge to lymph vessels which transport the lymph fluid through the draining lymph nodes. The thickness of the vascular wall increases due to the presence of connective tissue and smooth muscles. To transport the lymphatic fluid passively and to prevent its back flow, all the collecting lymphatics contain valves (Hawley *et al.*, 1995). The lymph vessels, except those from the right upper regions of the body, lead into the thoracic duct. It starts as a *pars abdominalis* in the abdominal cavity, parallel and partially dorsally of the abdominal aorta and is formed by the confluence of the mesenteric and lumbar collectors, including the unpaired *Cisterna chyli* (Ionac, 2003). Leading through the hiatus aortae of the diaphragm, the *pars thoracalis* of the duct passes through the thoracic cavity and enters the left cervical region. The thoracic duct represents the largest lymph vessel and empties into the blood circulation at the left jugular angle (Suami *et al.*, 2011). Except having thicker walls, the structure of the duct is similar to the collecting vessels. It consists of three layers. The intima has one layer of endothelial cells and a very thin *lamina elastica interna*. It is covered by the *media*, which has a layer of smooth muscle cells. In case of a strong filling phase of the *ductus thoracicus*, the *interna* and *media* are extended and flattened. The outermost layer is the *adventitia*. The inner portion of the adventitia is a mixture of taut collagenous connective

tissue and smooth muscles, which passes into an outer portion, formed by loose connective tissue (Azargoschasb, 1963).

In contrast to the rest of the body, the CNS is considered to lack anatomically defined lymphoid tissue. In 2013, the group of Nedergaard discovered a pseudo lymphatic function in the brain and named it the glymphatic system (Plog & Nedergaard, 2018). It is a waste clearance pathway of the brain and connected with an authentic lymphatic network of the *Dura mater*, which transports fluid into the deep cervical lymph nodes via foramina at the base of the skull (Benveniste *et al.*, 2019). While the exchange of substances is limited by the blood-brain barrier, other compounds such as large molecules, like peptides and proteins, must be cleared from the brain interstitium to the cerebrospinal fluid (CSF) (Iliff & Nedergaard, 2013). CSF is produced in the *plexus choroideus* and then flows into the subarachnoid space. From there, it enters the brain via perivascular space, surrounding the penetrating arteries, and distributes in the parenchyma (Jessen *et al.*, 2015). Controlled by the bulk flow, CSF exchanges with the interstitial fluid (ISF) of the brain. Then the ISF solute is drained back to the perivascular space surrounding the veins (Iliff & Nedergaard, 2013). This CSF-ISF exchange is facilitated by aquaporin-4 water channels, which are densely expressed within astrocytic processes. Astrocytes are glia cells and are located at the outer layer of the perivascular spaces (Plog & Nedergaard, 2018).

1.3 Physiology

The lymphatic system plays a key role in immune function, body fluid homeostasis, absorption and transport of dietary lipids, fat soluble vitamins and water insoluble compounds (McLennan *et al.*, 2005). It is a one-way transport system that gains direct access to the systemic circulation and is responsible for interstitial tissue fluid homeostasis (Zawieja *et al.*, 2019). Blood capillaries receive blood from the branching arteries and release plasma fluid and proteins into the interstitial space. Most of this exudate is reabsorbed, but a small amount is transferred through the interstitium into the lymph capillaries (Swartz, 2001). The protein composition of the lymph fluid stays nearly equivalent to the interstitial fluid, except the intestinal lymph, which has a higher concentration of fat. This difference is explained by the direct fat resorption from the intestine (O'Driscoll, 1992). In addition to the venous system it builds the second drainage system of the body (Swartz, 2001).

The lymph nodes also play an important role in the lymphatic system. On the way through the body the lymph fluid passes at least one of these structures (Morris & Courtice, 1977). Dendritic cells, macrophages, T- and B-cells (including plasma cells) are localized within the lymph nodes and develop their specific functions in specific and unspecific immune reactions (Moore Jr & Bertram, 2018).

Lymphocytes arise from the bone marrow. While T-lymphocytes leave the bone marrow to mature in the thymus, B-lymphocytes continue to mature in the bone marrow (Litwin, 2020). The naïve lymphocytes are in constant movement between blood circulation and lymphatic system (Morris & Courtice, 1977). These cells are able to leave the blood stream, migrate into tissue and return into the blood circulation with the lymph fluid, by passing the thoracic duct (Ford & Simmonds, 1972).

Biomarkers specific for lymphatic vessels, such as the vascular endothelial growth factor receptor (VEGFR)-3, the prospero homeobox (PROX) 1 transcription factor, the integral membrane glycoprotein podoplanin, the cluster of differentiation (CD) 31 and the lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 are commonly used to visualize lymphatic endothelial cells and lymph vessels (Natale *et al.*, 2017). The biomarkers made possible a complete understanding of lymphatic physiology, and furthermore are an important tool for investigation (Skobe & Detmar, 2000).

2 Lymphatic absorption

2.1 Intestinal lipid absorption

Compared to proteins and carbohydrates, lipids and other hydrophobic compounds contained in food, are almost insoluble in the aqueous digestive juices. Therefore enzymatic hydrolyzation requires special mechanisms (Ros, 2000). The digestive process of lipids, such as triacylglycerols, phospholipids and cholesteryl esters starts in the oral cavity with the lingual lipase, which is secreted by glands in the tongue, and is continued by the gastric lipase in the stomach (Iqbal & Hussain, 2009). Due to its peristalsis the stomach then represents the major site for the emulsification of dietary lipids and fat-soluble vitamins (Ros, 2000). In the duodenum, the fatty chyme mixes with the bile salts released via and with the pancreatic juice, which further hydrolyzes the lipids. The bile salts ensure an even finer emulsification for the formation of mixed micelles, while various pancreatic lipases continue hydrolyzation of the lipids in preparation for absorption across the intestinal wall (Mansbach & Gorelick, 2007). Emulsification and hydrolyzation of triacylglycerol result in the generation of free fatty acids and monoacylglycerols, while the digestion of phospholipids releases free fatty acids and lysophospholipids (Phan & Tso, 2001). Cholesteryl esters are hydrolyzed to free fatty acids and free cholesterol. In the aqueous environment of the intestinal lumen, these fat-soluble compounds are incorporated into bile acid micelles that deliver fatty acids and monoglycerides to the intestinal microvilli (Ros, 2000). Before passing through the cell membrane passively or by carrier-mediated diffusion, the lipolytic products are dissociated from micelles (Hussain, 2014). The uptake mechanism depends on the free fatty acid concentration in the intestinal lumen. If the concentration in the lumen exceeds those inside the cells, free fatty acids diffusion happens across the cell membrane of the enterocytes, while lower extracellular concentrations lead to an active protein-mediated transport (Iqbal &

Hussain, 2009). Within the epithelial cells, intestinal fatty acid binding proteins distribute these throughout the cytoplasm to the smooth endoplasmic reticulum, where the resynthesis of complex lipids to triglycerides occurs. In the rough endoplasmic reticulum these resynthesized triglyceride droplets are stored as intracellular lipid droplets or combine with 'primordial lipoproteins', consisting of phospholipids, apolipoproteins and cholesteryl ester (Trevaskis *et al.*, 2015b). These newly formed products, also called pre-chylomicrons mature in the Golgi apparatus and are exocytosed. Mature chylomicrons consist of a triglyceride and cholesterol ester core and of a phospholipid, cholesterol and apolipoprotein surface (Ko *et al.*, 2020). They are taken up by the mesenteric lymphatics and transported to the thoracic duct, which releases them into the blood circulation via the left venous angle. Lipoprotein lipase in the capillaries of fatty tissues and muscle tissues break down chylomicrons and consequently fatty acids are released and absorbed into the surrounding tissues (Ros, 2000).

2.2 Intestinal absorption of orally administered drugs

Oral administration is most convenient for patients and therefore the most preferred and widely used form of application (van Hoogdalem *et al.*, 1989). Thus, the understanding of oral bioavailability is a key step in drug development of both, newly developed and already existing drugs. While the majority of orally administered drugs gains access to the systemic circulation, highly lipophilic drugs are transported through the lymphatic system to the systemic circulation (Charman, 2000). Especially for macromolecules and poorly water-soluble drugs, the path of intestinal lymphatic drug transport is gaining in significance.

Orally administered small molecule drugs are directly absorbed and transit across the enterocytes of the small intestines into blood capillaries (Trevaskis *et al.*, 2008). This is the most common pathway because of the high rate of fluid flow in the portal blood, but at the same time it is restricted by tight junctions of the blood capillaries (Charman, 2000). Both, blood and lymph capillaries are densely distributed in the *lamina propria* of the intestines, in close proximity to the enterocytes (van Hoogdalem *et al.*, 1989).

Macromolecules like food derived lipids and fat-soluble vitamins, have a limited permeability through blood capillaries and utilize the more permeable lymphatic capillaries. Triglyceride rich lipoproteins formed by the chylomicrons in the endoplasmic reticulum incorporate the highly lipophilic drugs. The chylomicron-drug complexes are absorbed by the mesenteric lymphatics, after exocytosis via the Golgi apparatus (**Figure 2**) (Charman, 2000).

The advantage of the intestinal lymphatic absorption is the avoidance of the hepatic first pass effect resulting in an increase of oral bioavailability of highly lipophilic drugs (El-Kattan & Varma, 2012). While small molecule drugs, taken up by blood capillaries, are transported directly to the portal vein, lipophilic macromolecule drugs, absorbed via the intestinal lymph,

are carried to the thoracic duct, which is emptying into the blood circulation at the junction of the left jugular and subclavian veins (Managuli *et al.*, 2018).

In 1997, Lipinski defined with the 'rule of 5' the physicochemical requirements of drugs to achieve good oral bioavailability. The 'rule of 5' refers to a molecule having a molecular mass of less than 500 Dalton (Da) and an octanol–water partition coefficient log P not greater than 5. The primary absorption pathway for drugs with a log P > 5 and a solubility > 50 ng/g in long-chain triglyceride lipid is more likely the intestinal lymphatic system (Lipinski *et al.*, 1997).

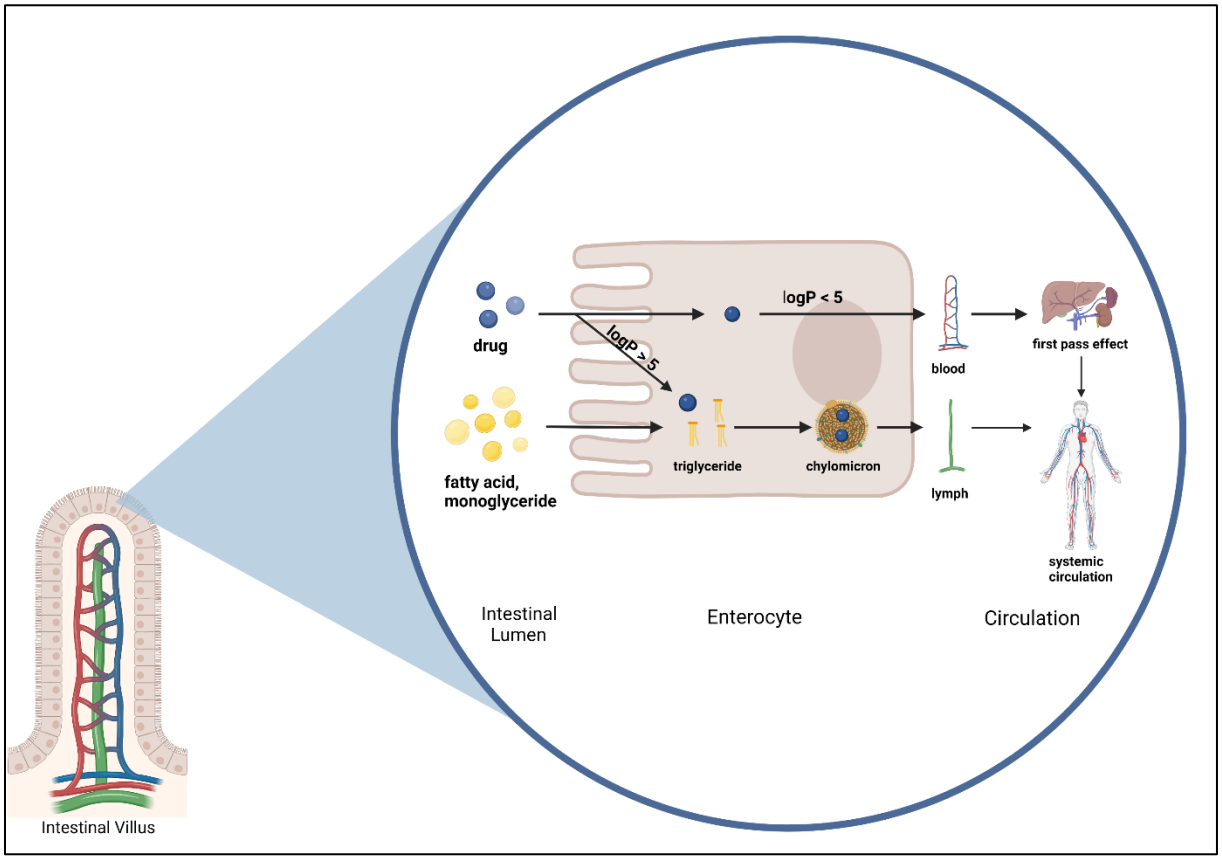


Figure 2: *Intestinal absorption of water-soluble drugs vs lipophilic drugs (created with BioRender.com).*

2.3 Lymphatic uptake and transport after subcutaneous injection

The subcutaneous (s.c.) injection is a commonly used form of administering a compound in humans and animals. In clinical use, it plays a very important role in the administration of macromolecules, hormones, and antibodies (Kagan et al., 2007). Because of the flow rate being approximately 100-500 times lower than in the blood, smaller shear stresses of the lymph vessels, and the presence and function of lymph nodes, this form of administration provides different advantages (Swartz, 2001). One advantage is that it allows less frequent administration compared to intravenous (i.v.) dosing, thus improving patients' convenience, which is explained by a delay in the time in which the maximum plasma concentration is reached and by a prolonged terminal half-life (McLennan et al., 2005). While small drug molecules are able to diffuse paracellularly or transcellularly through the vascular endothelia,

absorption of macromolecules by the blood capillaries is limited due to their tight junctions. For these molecules, the lymphatic system provides an alternative absorption pathway (Torres-Terán *et al.*, 2021). Factors affecting the absorption of substances administered subcutaneously begin with the site of application and the respective construction of the interstitium (Kagan, 2014). The interstitium consists of collagen, glycosaminoglycans and elastin which form a gel like, negatively charged milieu. It gives the extracellular matrix a structure to maintain its volume and leads to an electrostatic interaction with the charge of macromolecules transported through the interstitium (Scallan, Huxley, & Korthuis, 2010). The densely distributed, blind-ended lymphatic capillaries absorb the macromolecules from the interstitial space. They transport the lymph, containing the subcutaneously administered drugs, to the systemic circulation. On this way they are passing the larger collecting vessels, lymph nodes and the *ductus thoracicus* (McLennan et al., 2005).

Previous studies already showed the influence of different factors such as size, charge, hydrophilicity, solubility and formulation characteristics of a molecule affecting the interstitial diffusion (Kagan, 2014). However, the mechanism of subcutaneous absorption is not fully understood, including the incomplete bioavailability and requires further investigation.

3 Immunology

3.1 Cytology of the lymph

Despite the importance of the lymphatic system there are only a few reports describing the detailed cellular composition of the lymphatic fluid (Reinhardt & Yoffey, 1957; Azargoschasb, 1963; Morris & Courtice, 1977; Johnson & Jackson, 2008): The lymph cellular fraction is predominantly composed of immune cells and some apoptotic cells (Hansen *et al.*, 2015). Tissue-derived antigens, dendritic cells and lymphocytes are transported from tissues to draining lymph nodes (Trevaskis *et al.*, 2015a). The immune response is regulated by the antigen presenting cells that present soluble antigens to lymphocytes located in the lymph nodes (Santambrogio, 2018). Lymphatic endothelial cells promote this process actively with the coordinated increased expression of chemokines and adhesion molecules shaping the adaptive immune response through influencing immune cell trafficking (Card *et al.*, 2014).

The number of white cells in lymphatic fluid varies between 6.200 and 15.400 per mL (10.000 per mL on average) (Azargoschasb, 1963). The majority of the cells are lymphocytes that originate from precursor cells in the bone marrow (Morris & Courtice, 1977). The lymphocytes in the lymph fluid can be categorized as large lymphocytes with a more central and rounder nucleolus and small lymphocytes with eccentric nucleolus and constrictions (Reinhardt & Yoffey, 1957). Other cells in the lymphatic fluid are monocytes, lymphoblasts, plasma cells and mast cells (Azargoschasb, 1963).

3.2 The migration of dendritic cells to primary skin-draining lymph nodes

The epidermis and dermis of the skin which are separated by a basal membrane, play an important role in the immune defense triggered by environmental pathogens (Förster *et al.*, 2012). The epidermis consists of a lipid-rich intercellular substance that forms a water repellant layer, and of a layer of keratinocytes connected via tight junctions (Roediger *et al.*, 2008). Also located in the epidermis are skin resident immature dendritic cells, the so-called Langerhans cells, that penetrate these tight junctions with protruding dendrites to actively collect antigenic material by endocytosis (Randolph *et al.*, 2005). With the ability of activated Langerhans cells to penetrate the basal membrane they enter the dermis. Within the dermis, resting dendritic cells are activated and migrate towards the blind ending lymph vessels. The activated dermal dendritic cells are able to enter the lymphatic system by passing through the uninterrupted channels of the endothelial cells (Girard *et al.*, 2012). After trafficking actively to the larger lymph vessels, the dendritic cells are transported passively via the lymph flow to the primary skin-draining lymph nodes (Roediger *et al.*, 2008).

3.3 Cell trafficking through lymph nodes

Lymph nodes are highly organized, bean-shaped structures that are connected to the blood circulation as well as to the lymphatic system (Girard *et al.*, 2012). They occur individually, in groups or as chains of knots along the lymphatic vessels and serve as a filter for the lymphatic fluid. They play an important role in the treatment of immune-related diseases or preventive treatments, such as vaccinations as they provide a site for immune surveillance and generation of immune responses (Trevaskis *et al.*, 2015a). The structure of lymph nodes can be divided into three parts, the cortex, paracortex and medulla. The cortex is located closest to the capsule (Girard *et al.*, 2012). It is also named B-zone, as B-lymphocytes differentiate and proliferate in this part of the lymph node. The paracortex, also called T-zone, is surrounded by the cortex and medulla (Elz *et al.*, 2021). Here mainly T-lymphocytes are located and activated. Additionally, specialized high endothelial veins (HEVs) within the paracortex allow naïve T- and B-lymphocytes to extravasate from the blood and to enter the lymph nodes (Girard *et al.*, 2012). The medulla of the lymph node is located next to the hilus of the lymph node. Linearly arranged reticulum cells as well as short-lived plasma cells and macrophages form medullary cords. This is where the antigen-dependent affinity maturation of B-lymphocytes is taking place (Salomon *et al.*, 2015).

Afferent lymph vessels enter through the capsule of the lymph nodes and transport antigen presenting dendritic cells, memory T-cells and macromolecules from the periphery (Randolph *et al.*, 2005). The lymph fluid entering the lymph node is called primary lymph (Salomon *et al.*, 2015). A subcapsular sinus along the capsule of the lymph node can forward a part of the lymphatic fluid, which is not entering the lymph node parenchyma, directly towards the hilus, the origin of the efferent lymph vessel (Girard *et al.*, 2012). Alternately, larger particles are typically phagocytosed by subcapsular macrophages while smaller particles or small molecules

enter the B- or T-cell zones through lymphatic sinuses. These sinuses are forming a mesh from the subcapsular sinus through the cortex and paracortex and unite at the medullary sinus (Trevaskis *et al.*, 2015a). Within this mesh small molecules are taken up by B-cells and antigen-presenting dendritic cells which interact with T-cells. Crossing the lymphatic sinuses several times ensures a successful interaction that enables immune responses to potential foreign particles (Elz *et al.*, 2021). After this process secondary lymph with naïve lymphocytes is passively carried away through the medullar sinus into the efferent lymph vessel (Salomon *et al.*, 2015). Before entering the blood circulation via the thoracic duct lymph fluid is passing through several downstream lymph nodes (Trevaskis *et al.*, 2015a).

3.4 Simplified T-cell activation

T-cells are lymphocytes that are generated in the bone marrow, thymus and extrathymic tissue (Shimizu *et al.*, 1999) and have a T-cell receptor (TCR) on their cell surface. Depending on their subtype, they have different important functions in the adaptive immune response (Pennock *et al.*, 2013). While CD 8+ T-cells differentiate to T-killer cells, CD4+ T-cells mature to regulatory cytokine and chemokine producing T-helper cells (Alberts *et al.*, 2002). In the blood circulation T-lymphocytes bind with their TCR to the major histocompatibility complex (MHC)-complex of antigens, presented by antigen presenting cells such as dendritic cells. The TCR is composed of one α and one β chain and a CD3 complex constituted of γ-, δ-,ε- and ζ-membrane proteins (**Figure 3**). Depending on the subtype, the TCR features a CD4 or CD8 co-receptor, that clusters with the MHC proteins (Smith-Garvin *et al.*, 2009). By binding of the MHC-complex, the CD3 complex transduces the first activation signal into the cell interior. Inside the T-cell, Lck, a Src-like cytoplasmic tyrosine kinase, phosphorylates on the ζ and ε chains of the CD3 complex (Alberts *et al.*, 2002). This activates and phosphorylates ZAP-70, another cytoplasmic tyrosine kinase, which binds to Lck and in turn phosphorylates the transmembrane adaptor molecule linker for activation of t-cells (LAT) (Pennock *et al.*, 2013). The phosphorylated LAT generates a multiprotein complex consisting of a number of signaling molecules at the plasma membrane known as the "LAT signalosome" (Richard *et al.*, 2021). In addition, a co-receptor protein CD28 on the surface of the T-cell recognizes costimulatory proteins like CD80 and CD86 on the antigen presenting cells and induces a second signal (Alberts *et al.*, 2002). These two signals stimulate the secretion of interleukin 2, a cytokine that binds to simultaneously synthetized interleukin 2 receptors. This activates an intracellular signaling pathway and helps the T-cell to proliferate and differentiate (Pennock *et al.*, 2013).

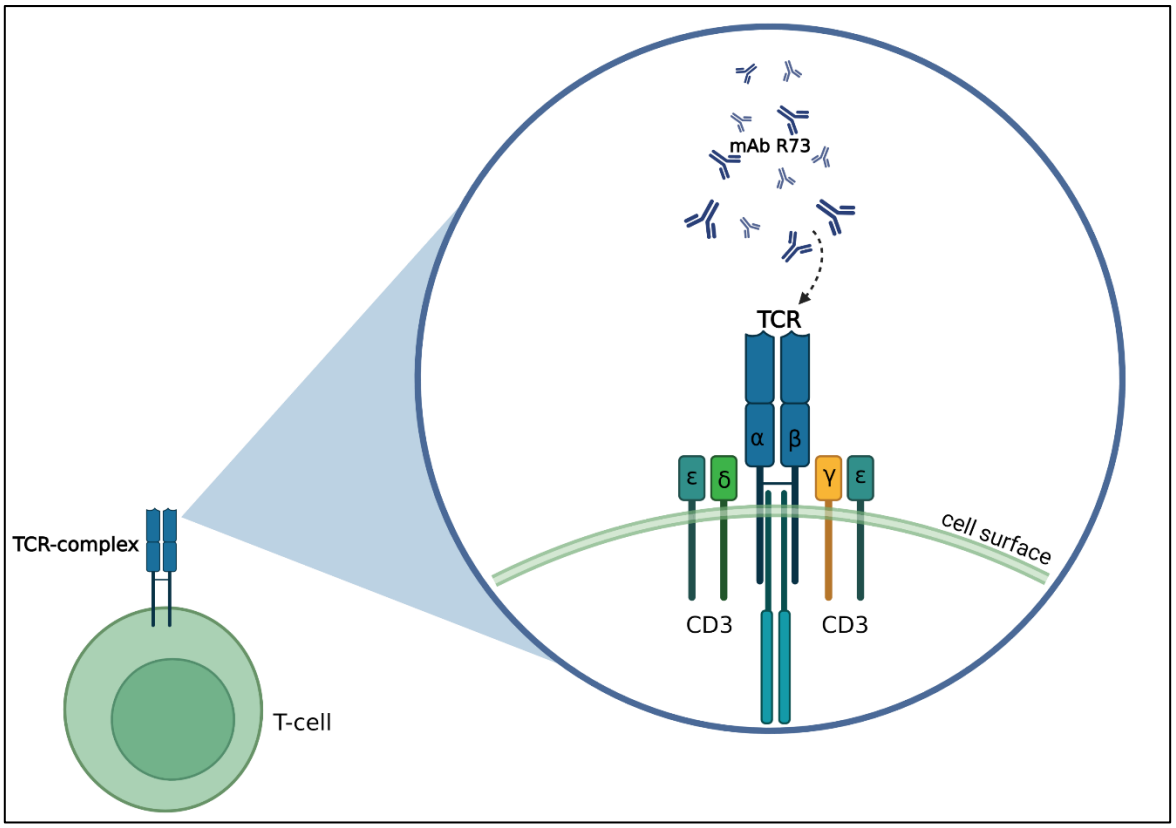


Figure 3: *Simplified T-cell receptor (TCR), binding site for the monoclonal antibody mAb R73 (created with BioRender.com).*

3.5 Simplified B-cell activation

B-cells develop in the bone marrow from lymphoid progenitor cells and mature into pre-B-cells. During the maturation process, a B-cell receptor complex (BCR) develops, which is composed of membrane-bound immunoglobulins, immunoglobulin (Ig) M and IgD (Paul, 2012). This BCR allows differentiation from other lymphocytes along with additional CD markers (Cano & Lopera, 2013). By leaving the bone marrow, the mature B-cells have not had any contact to antigens yet and are thus considered as naïve B-cells. They circulate as inactive cells in the blood circulation and secondary lymph organs (Pezzutto *et al.*, 2007). For the activation of B-cells, a distinction is made between T-cell dependent and T-cell independent activation. As the name suggests, the help of T-helper cells is necessary for T-cell dependent activation. It takes place in the secondary lymphatic organs. Once an antigen binds to the BCR, the antigen is endocytosed (Cano & Lopera, 2013). It is then presented again together with an MHC molecule on the cell surface. The T-helper cell activates the B-cell by binding to this antigen-MHC complex and releasing cytokines. Upon activation, the B-cell proliferates and differentiates into a plasma cell. In addition, antibodies of the class IgG, IgA and IgE, as well as memory-B-cells are formed (Paul, 2012). In the case of T-cell independent activation, the B-cell binds with its BCR to the antigens and is activated, proliferates and forms IgM antibodies, only. While T-cells only bind to the peptide fragments of the antigen presented as an antigen-MHC complex, B-cells bind to antigens directly (Pezzutto *et al.*, 2007).

4 Description of the medication used as tool compounds

4.1 Compound 1 – Vitamin D_3

Vitamin D_3, also called Cholecalciferol, is an endogenous fat-soluble vitamin and belongs to the seco-steroids (Demer *et al.*, 2018). Because of its poor water solubility Vitamin D_3 is formulated in oil solutions or oil-on dry carriers in form of drops, capsules, tablets and oil-based injectables (Hirsch, 2011). Beside the plant derived Vitamin D_2 ,Vitamin D_3 is a form of Vitamin D which is used in the treatment of specific medical conditions such as disturbed bone mineralization, muscle weakness, hypoparathyroidism as well as chronic kidney disease (Momekov *et al.*, 2016). It is generated in the skin of mammals and can be ingested with various diets or health supplements (Demer *et al.*, 2018). It is absorbed from the small intestine, linked to fat absorption facilitated by the secretion of bile and pancreatic enzymes. Within the enterocytes, it is bound with triglycerides in chylomicrons and transported via the lymphatic system to the blood circulation (Dahan & Hoffman, 2005).

Property Name	**Property Value**
Molecular Formula	$C_{27}H_{44}O$
Molecular weight	384.6377
Log P	7.13
Hydrogen Bond Donor Count	1
Hydrogen Bond Acceptor Count	1
pK_a	18.38

Table 1: *Basic pharmacokinetic data for compound 1 (from internal database).*

4.2 Compound 2

Compound 2 is a B-cell lymphoma (Bcl)-2 family protein inhibitor that is being developed as a potential agent for the treatment of cancer. It is an orally bioavailable, synthetic small molecule, which binds to apoptosis suppressor proteins Bcl-2, Bcl-XL and Bcl-w and displays potent mechanism-based cytotoxicity against human tumor cell lines derived from small cell lung carcinomas and lymphoid malignancies (both T-cell and B-cell origin). Among existing chemotherapeutics, cytotoxic agents represent a key component of most current therapeutic regimens. These anti-cancer agents trigger the cell to undergo apoptosis, by inhibiting the overexpression of pro-survival proteins such as the Bcl-2 family.

Property Name	Property Value
Molecular weight	991.08
Log P	8.163
Hydrogen Bond Donor Count	2
Hydrogen Bond Acceptor Count	11
pK_a	4.26

Table 2: *Basic pharmacokinetic data for compound 2 (from internal database).*

4.3 Compound 3 – Itraconazole

Itraconazole is a third generation synthetic antifungal triazole imidazole, first synthesized in 1980 (Van Cauteren *et al.*, 1987). It is well-tolerated, has a broad-spectrum activity and is the only antifungal agent with oral bioavailability, effective against the most common fungal pathogens such as Blastomycosis species, Histoplasmosis species, Candida species and Aspergillus species (De Beule & Van Gestel, 2001).

Azoles act by altering the cellular membranes of susceptible fungi. Itraconazole has three nitrogen atoms in its five-membered azole ring, which interact with the ferrous ion of the heme of the fungal cytochrome P4503A (Prentice & Glasmacher, 2005). This leads to an inhibition of 14α-demethylase, an enzyme, that converts lanosterol to ergosterol and is required in fungal cell wall synthesis. Instead of ergosterol, lanosterol and different 14α-methyl sterols accumulate within the cell membrane (Riviere & Papich, 2018). Impairment of ergosterol synthesis results in increased cellular permeability causing leakage of cellular components, alterations in membrane-bound enzyme activity and the coordination of chitin synthesis (De Beule & Van Gestel, 2001).

Itraconazole is available in capsules, as an oral solution or as liquid dosage form for intravenous administration. A reason for the different forms of formulation is the inconsistent absorption caused by the pharmacochemical properties of the drug (Grant & Clissold, 1989; Lestner & Hope, 2013). Itraconazole is a very weak base, being practically un-ionized at physiological pH. The solubilization of the almost water insoluble and highly lipophilic drug might be a rate-limiting step in gastrointestinal absorption that can be increased by an acid environment, such as in gastric secretion (Bae *et al.*, 2011). Administering Itraconazole capsules directly after food uptake or in combination with an acidic beverage may improve the systemic availability (De Beule, 1996).

To improve oral bioavailability, the Itraconazole oral solution contains hydroxypropyl-ß-cyclodextrin. These are substituted glucose molecules arranged in a closed circle with a cylinder like structure. Because of a lipophilic central cavity and a hydrophilic outer surface these rings are able to form non-covalent inclusions complexes with lipophilic drugs like Itraconazole (Rasheed, 2008). The cyclodextrin-itraconazole complexes have an improved oral

bioavailability compared to the Itraconazole capsules because of the absence of the dissolution process on delivery to the stomach (De Beule & Van Gestel, 2001).

Because of its lipophilia, Itraconazole is highly bound to plasma proteins, especially albumin and red blood cells, resulting in an extensive distribution throughout the body (Riviere & Papich, 2018).

Property Name	**Property Value**
Molecular Formula	$C_{35}H_{38}Cl_2N_8O_4$
Molecular weight	705.65
Log P	7.31
Hydrogen Bond Donor Count	0
Hydrogen Bond Acceptor Count	9
pK_a	3.7
Protein binding	> 98 %

Table 3: *Basic pharmacokinetic data for compound 3 (from internal database).*

4.4 Compound 4 – R73 monoclonal antibody (mAb)

R73 is a purified mouse monoclonal antibody, which is specific for rat α/β T-cell receptor of most peripheral mature and immature T-lymphocytes, intestinal intraepithelial lymphocytes, and thymocytes (BD Biosciences Web Page). The TCR is a heterodimer, membrane-bound protein complex. It consists of an alpha and a beta peptide chain, linked by a disulfide bridge, and forms an antigen binding site in the extracellular space. It binds to an epitope of the regular area of the rat T-cell receptor and induces detectable rat T-cell activation and differentiation in the blood (Hünig *et al.*, 1989). In previous *in-vivo* studies the treatment with mAb R73 showed a reduction of severe experimental autoimmune reactions, transplant rejection, and graft-versus-host response (BD Biosciences Web Page).

IV. WORKING HYPOTHESES AND AIMS

In preclinical drug development investigation of factors influencing the absorption, distribution, metabolism and excretion (ADME) profile of molecules in order to identify the optimal drug and formulation properties for human use is a key challenge. It is assumed that the physicochemical properties of a drug as well as its dosage form and formulation are important factors that can influence the absorption pathway and that determine bioavailability of the drug. Therefore, this thesis is intended to investigate the lymphatic uptake and distribution of medications, the influence that modifications of the formulation have on the lymphatic uptake, and potential drug actions within the lymphatic system. In a first step of this work, an *in-vivo* surgical method of cannulating the thoracic duct should be established to allow investigation of lymphatic uptake and distribution of either orally or subcutaneously administered drugs. Using this approach, factors that may positively influence the bioavailability of these molecules should be determined. In addition, for subcutaneously administered large molecules, it should be shown how they enter the circulation from the subcutis via the lymph nodes and vessels, and how their potential interactions with cells might influence the immune system.

In particular, this work aims to answer the following questions:

1. *Can an animal model for the collection of lymph be established?*

2. *Are the orally administered compounds used here absorbed via the lymphatic system?*

3. *Can the oral bioavailability of orally administered compounds be improved by administration of lipid based formulations*?

4. *Has olive oil administration an effect on the oral bioavailability?*

5. *Is Fluorescence Activated Cell Sorting (FACS) analysis suitable for the analysis of lymphatic fluid?*

6. *Is the subcutaneously administered immunology compound absorbed and distributed via the lymphatic system?*

V. MATERIAL AND METHOD

1 Literature research

Literature from public resources

The initial literature review was conducted in January 2020 and was carried out continuously throughout the completion of this thesis. The extensive literature search was performed in the following databases with the key words or their combination, for example:

„Cannulation thoracic duct“/ „Lymph collection rats“/ „Thoracic duct cannulation rat“/ “ Lymph absorption in rats“/ “Lymphatic transport of drugs“/ “Cannulation technique thoracic duct“/ ”Lymphatic system rat model”/ ”Lymphatic transport anesthetized rat model”/ ”Oily vehicles lymphatic system in rats”/ ”Visualization thoracic duct rat”/ ”Surgical method lymph cannulation”/ ”Lymph cannulation surgical procedure”/ ”Thoracic duct in rats”/ “Intestinal lymphatic absorption”/ “Cell composition in lymph”/ “Rule of five”/ “T-cell activation”/ “Lymphocytes”/ “Vitamin D_3”/ Lymphatic absorption of vitamin D_3”/ Bcl-2 inhibitors”/ “Itraconazole”/ “mAb 73”/ “Subcutaneous lymphatic absorption” and variations of them.

Databases: Google Scholar® PubMed®, go3R®

Additional references were found by searching the reference list of relevant publications.

Literature from internal resources

Planning the experiment, experts who have experience with microsurgical operations on rodents (microdialysis, cannulation of vessels) were first contacted. The technique was demonstrated and internally trained by an external surgeon. The technique was further refined by continuous training. For information on compounds internal databases were used.

2 Animal welfare

2.1 Animal research allowance

The responsible authority (Landesuntersuchungsamt Rheinland-Pfalz, Referat 23, Mainzer Straße 112, 56068 Koblenz) approved AbbVie animal research allowance with the title “Untersuchung von Aufnahme und Verteilung von Arzneimitteln über die Lymphe” (translated: “Investigation of uptake and distribution of drugs via the lymph”) in April 2021. The request was approved under the reference number: 23 177-07/G 21-9-006. The approved

substance requests are listed under the reference numbers: 23 177-07/G 21-9-006 E2&E3 and 23 177-07/G 21-9-006 E4.

Study design and conduct have complied with the following laws of the Federal Republic of Germany:

- Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes (published in German Federal Gazette 2013, Part I, Nr. 47).

- Ordinance on the protection of animals used for experimental or scientific purposes published on 01 August 2013 (German implementation of EU directive 2010/63; BGBl. I S. 3125, 3126).

- Commission Recommendation 2007/526/EC on guidelines for the accommodation and care of animals used for experimental and other scientific purposes published on 18 June 2007.

- The German Animal Welfare Act as published on 18 May 2006 (BGBl. I S. 1206, 1313), amended according to Art. 20 G from 09 December 2010 I; amended by Art. 280 V v.19 June 2021 I 1328.

- Environment Directorate, Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology. Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Human Endpoints for Experimental Animals in Safety Evaluation. ENV/JM/MONO (2000)7. OECD, 2000.

In addition, AbbVie is certified by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Accreditation sets international standards for animal care and ensures animal well-being in research.

2.2 Animal housing and environment

The animals were cared for in accordance with the principles and guidelines of the German Animal Welfare Act.

Environmental conditions

Upon arrival, rats were allowed to acclimate for at least five days. The laboratory animals were housed two or three per cage in Type IV cages (polysulfone or polyetherimide) with bedding

(dust-free softwood shavings; ssniff GmbH; 59494 Soest, Germany) in a 12-h dark/light cycle. As enrichment wood and nesting material was placed in the animal cages. The room temperature was 22 °C ± 2 °C (= 20 – 24 °C) with a relative humidity 55 +/- 10 % (45 – 65 %). Both parameters are recorded continuously by a digital Monitoring System (Pulse CMC™ Software, Lab Monitoring System - Avidity Science). During the acclimatization period rats were fed *ad libitum* with standard dry food (ssniff Rodent Maintenance 2014 pellets; ssniff Spezialdiäten GmbH, 59494 Soest, Germany) and had 24 hours free access to water, provided in bottles with a stainless-steel drinking nipple. Changes were made in the feeding regime only for conduction of experiments. These are described later (**Chapter 7**).

2.3 Veterinary care

From the time rats arrived in the facility, the laboratory animals are supervised at least twice a day on weekdays and once a day on weekends by animal technicians. On days without experiments at least one veterinarian was on site during the week or on-call during weekends. General veterinarian examinations were done three days after arrival, during animal selection and prior to surgeries and dosing days of satellite animals. Satellite animals are extra animals dosed as per protocol for the evaluation of pharmacokinetic characteristics of the compound only. The surgical experiments were performed by a veterinarian. For satellite animals, score sheets (**Appendix, Section 1**) were provided and filled out in the event of animals presenting clinical signs, to determine the criteria for consulting a veterinarian or for meeting the termination criteria. No adverse clinical signs were observed throughout all experiments and therefore no animal was euthanized ahead of scheduled termination. Only healthy animals were used for the experiments. Hygiene monitoring is carried out according to Federation for Laboratory Animal Science Associations (FELASA) recommendations via the quarterly examination of litter sentinels (Mähler *et al.*, 2014). Additionally health reports of the animals are available from the suppliers.

3 Study planning, experimental animals, animal selection and identification

3.1 Study planning

All studies were planned according to the Reporting of Recommendations for Excellence (PREPARE). For each study an internal study plan was written. For the writing of the thesis the Planning Research and Experimental Procedures on Animals: In Vivo Experiments (ARRIVE) and Systematic Review Center for Laboratory animal Experimentation (SYRCLE Risk of Bias Tool) were followed.

3.2 Animal selection

The current state of scientific knowledge does not provide acceptable alternatives, *in-vitro* or otherwise, to the use of live animals to accomplish the purpose of this study. Although the method has already been described in mice, it was considered to be unsuitable due to the small body size of mice and the associated higher failure rate (Banan *et al.*, 2021); therefore, 7 to 8-week-old, male, naïve rats were used for the experiments. Similar to Drug Metabolism and Pharmacokinetics (DMPK) studies, only one sex was used for the experiments, considering the 3 R (replacement, reduction and refinement) rules to keep the number of animals as low as possible. Male animals were selected based on available comparative data from previous studies. For pharmacokinetic and toxicological study types, rats are recommended by the authorities in the corresponding guidelines and recognized by regulators. This animal species is regularly used in toxicological and pharmacokinetic studies. A comprehensive database is available for the substances to be examined, which enables the scientific assessment of the results. In addition, the experiment was approved by the authorities in dogs, with the obligation to establish the method in rodents first.

3.3 Experimental animals

For the studies, male Sprague-Dawley [Crl: CD® (SD)] from Charles River Laboratories Germany, 97633 Sulzfeld, Germany were used. The animals arrived at least 5 days prior to their scheduled surgery date, and were pair or grouped housed, with a maximum of 3 animals per cage. At the initiation of the study they were expected to have a weight in the range of 200-350 g and to be 7 to 8 weeks old. To ensure the rats were approximately the same body weight, animals were ordered individually for each experimental week. On dosing days, rats undergoing surgery for the lymphatic cannulation were monitored using the Rodent Surgical Monitor+ and Indus Instruments. Pulse, respiration, blood pressure and body temperature were measured continuously while the animals were anesthetized.

Satellite rats were supervised at blood collection time points. The direct connection between the lymphatic system and the blood circulation is interrupted by the cannulation of the thoracic duct. Therefore, the amount of compound absorbed via the lymphatic system cannot reach the blood circulation. In order to be able to measure the total plasma level of the compounds, satellite groups were included.

3.4 Animal identification

To individually identify the rats, they were implanted with a IMI500 Transponder (BMDS Lab Animal Identification) two days after arrival. All animals were entered in the animal house database Tick@lab and assigned a batch identification number (batch ID). Individual cage cards in front of each cage provided additional information about sex, species, strain, breeder, date of birth, responsible personnel, study number, allowance reference number etc.

During the study, batch IDs were replaced with study numbers “Tier 1 to Tier 36” written on the tail with a permanent marker. Rats were randomly assigned to the different formulations and surgical- or satellite groups, using Excel (**Table 4**). The animals were randomized using the “ZUFALLSZAHL” (translated "RANDOM NUMBER") function by assigning them a random number between 0 and 1. With the help of an Excel Macro button, the animals were automatically copied into the pre-created schedule. Following the same principle, a formulation was assigned to the experimental days in another Excel file. Since the experiments were performed in a blinded manner, the allocation of the formulations and later also the administration of the formulations were carried out by an additional laboratory technician. The samples were only unblinded after the analysis of the samples was completed.

IA22-007 Randomisierung:										
1. Operationswoche:		Batch ID:								
Tier 4	0.86230424	74490		Tag 1	Tier 4		Randomisieren			Surgery
Tier 5	0.19663581	74491		Tag 2	Tier 5					Satellite
Tier 2	0.80923109	74488		Tag 3	Tier 2					
Tier 3	0.30033282	74489		Tag 4	Tier 3					
Tier 1	0.12155014	74487		Tag 5	Tier 1					
2. Operationswoche:										
Tier 8	0.5189687	74735		Tag 6	Tier 8		Randomisieren			
Tier 7	0.76436701	74734		Tag 7	Tier 7					
Tier 6	0.53267755	74733		Tag 8	Tier 6					
Tier 10	0.6988192	74737		Tag 9	Tier 10					
Tier 9	0.82241852	74736		Tag 10	Tier 9					
3. Operationswoche+ Satellitentiere:										
Tier 23	0.69727174	75201		Tag 11	Tier 23		Randomisieren			
Tier 27	0.04770326	75205		Tag 12	Tier 27					
Tier 17	0.72285754	75195		Tag 1 (Satellit1)	Tier 17					
Tier 11	0.82352414	75189		Tag 2 (Satellit1)	Tier 11					
Tier 21	0.92506839	75199		Tag 3 (Satellit1)	Tier 21					
Tier 19	0.33198292	75197		Tag 4 (Satellit1)	Tier 19					
Tier 15	0.87550517	75193		Tag 5 (Satellit1)	Tier 15					
Tier 24	0.56316145	75202		Tag 6 (Satellit1)	Tier 24					
Tier 13	0.18898268	75191		Tag7 (Satellit1)	Tier 13					
Tier 16	0.57244088	75194		Tag 8 (Satellit1)	Tier 16					
Tier 30	0.99509124	75208		Tag 9 (Satellit1)	Tier 30					
Tier 14	0.4006353	75192		Tag 10 (Satellit1)	Tier 14					
Tier 20	0.24371535	75198		Tag 11 (Satellit1)	Tier 20					
Tier 29	0.96768682	75207		Tag 12 (Satellit1)	Tier 29					
Tier 28	0.52351826	75206		Reservetier	Tier 28					
Tier 25	0.00727507	75203		Tag 1 (Satellit 2)	Tier 25					
Tier 33	0.30965337	75211		Tag 2 (Satellit 2)	Tier 33					
Tier 12	0.14028893	75190		Tag 3 (Satellit 2)	Tier 12					
Tier 26	0.87777356	75204		Tag 4 (Satellit 2)	Tier 26					
Tier 22	0.06563696	75120		Tag 5 (Satellit 2)	Tier 22					
Tier 32	0.30996223	75210		Tag 6 (Satellit 2)	Tier 32					
Tier 31	0.69142787	75209		Tag 7 (Satellit 2)	Tier 31					
Tier 18	0.55786302	75196		Tag 8 (Satellit 2)	Tier 18					
Tier 34	0.85159382	75212		Tag 9 (Satellit 2)	Tier 34					
Tier 35	0.04354357	75213		Tag 10 (Satellit 2)	Tier 35					
Tier 37	0.62732943	75215		Tag 11 (Satellit 2)	Tier 37					
Tier 36	0.65258854	75214		Tag 12 (Satellit 2)	Tier 36					

Table 4: *Rats assigned randomly to experimental day and groups.*

4 The surgery - general consideration

To enable aseptic work, only sterile disposable items or instruments autoclaved before surgery were used. Because of their very delicate structure and often almost transparent appearance, the lymph vessels, including the thoracic duct, are very hard to see. The lymph can be visualized by any lipid, as they have the property to allow the lymph fluids to appear in a white-milky color. To improve the anatomical visualization, the animals received 0.1 mL of olive oil by oral gavage 1.5 – 2 hours before dosing.

4.1 The surgical equipment

Instruments

Instruments:	**Model/ manufacture:**
Basic scissors	Aesculap, chirurgische Schere, BC771R, B. Braun Melsungen AG
Dissection scissors	METZENBAUM DUROTRIP Hartmetall Präparierschere, BC274R, B. Braun Melsungen AG
Surgical forceps	Aesculap, chirurgische Pinzette, BD552R, B. Braun Melsungen AG
Curved anatomical forceps	Graefe Forceps - Curved / Serrated, 11051-10, Fine Science Tools GmbH
Octagon forceps	Octagon forceps, 11041-08 Fine Science Tools GmbH
Hemostat	HALSTED Arterienklemmen, BH202R, B. Braun Melsungen AG
Micro needle holder with flat handle	CASTROVIEJO Nadelhalter, gebogen, BM564R B. Braun Melsungen AG
Needle holder	HEGAR-OLSEN DUROGRIP Hartmetall Nadelhalter, BM124R, B. Braun Melsungen AG

Table 5: *The surgical instruments.*

Other materials

Materials:	**Model/manufacture:**
Catheter (Thomafluid LDPE-Schlauch)	Polyethylene, transparent, ID:0.4 mm OD:0.8 mm VWR International GmbH
Retraction system	Retraction Kit for Small Animals 200 g to 5 kg, Fine Science Tools GmbH
Rodent Surgical Monitor+	Rodent Surgical Monitor+, Indus Instruments company Units: Heated Surgical Platform, Temperature Probe and 9.7” Touchscreen Display Unit.
Microscope	LEICA Stereomikroskop M80 Leica Microsystem GmbH

Objective	0.5 WD 188,5 mm, ACHRO 0.5x Leica Microsystem GmbH
Shaver	Aesculap Isis GT 420 Aesculap AG
Syringes	-Omnifix ® Solo Spritzen 2 mL, B. Braun Melsungen AG -Omnifix®-F Tuberkulin 1 mL, B. Braun Melsungen AG
Needles	-18G/23G/G Sterican Einmal-Injektions-Kanülen B. Braun Melsungen AG - Micro-Kanüle 25 G SARSTEDT AG & Co. KG
Tissue sterile drapes	Foliodrape 45 cm x 75cm, PAUL HARTMANN AG
Sterile gauze	Gauze Swabs 5 x 5 cm, absorbent cotton gauze, Wilhelm Weisweiler GmbH & Co. KG
Wound dressing	Solvaline N 10x10 cm, wound dressing, low adherent Lohmann& Rauscher International GmbH
Sterile cotton sticks	- Wattestäbchen, Heinze Herenz Medizinbedarf GmbH - Sterile swabs, wood + cotton, Deltalab company
Surgical glue	Surgibond surgical tissue adhesive 2.5 g + 7 applicators SMI AG
Suture material	- PH-Seide SCHW GEFL 6-0, 0.30, 711H, Johnson & Johnson Medical GmbH - Vicryl violett geflochten 3-0, V316H, Johnson & Johnson Medical GmbH
Lithium-Heparin tube	Micro sample tube with attached push cap 1.3 mL, SARSTEDT AG & Co. KG
EDTA tube	Microvette® CB 300 K2E, SARSTEDT AG & Co. KG

Table 6: *Additional necessary surgical materials.*

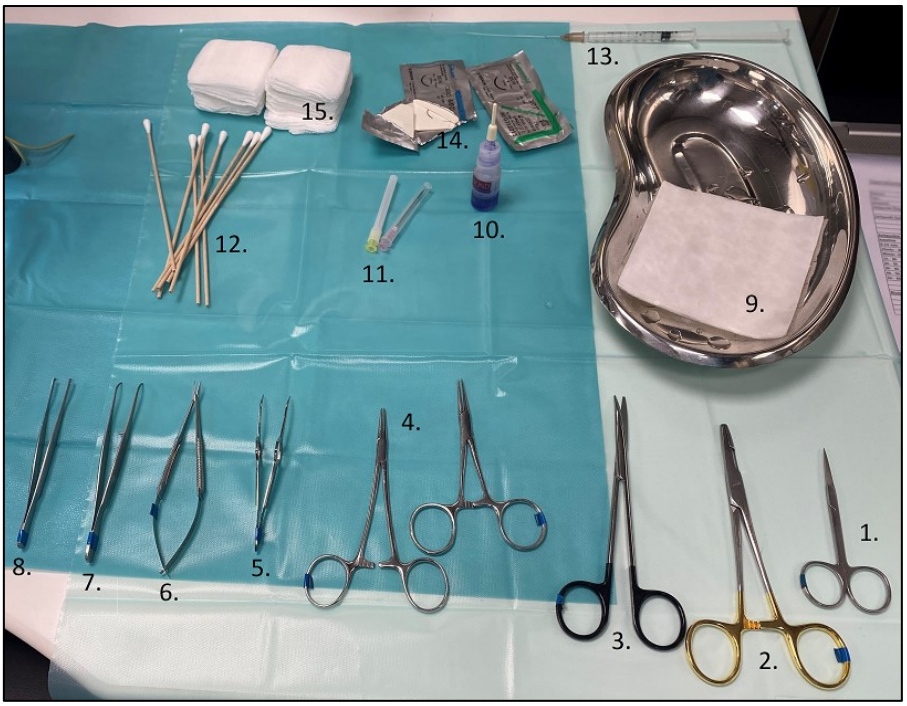


Figure 4: *The surgical equipment: (1) basic scissors, (2) needle holder, (3) dissection scissors, (4) hemostat, (5) octagon forceps, (6) micro needle holder with flat handle, (7) curved anatomical forceps, (8) surgical forceps, (9) wound dressing, (10)surgical glue, (11) needles, (12) sterile cotton sticks, (13) heparinized saline, (14) suture material, (15) sterile gauze.*

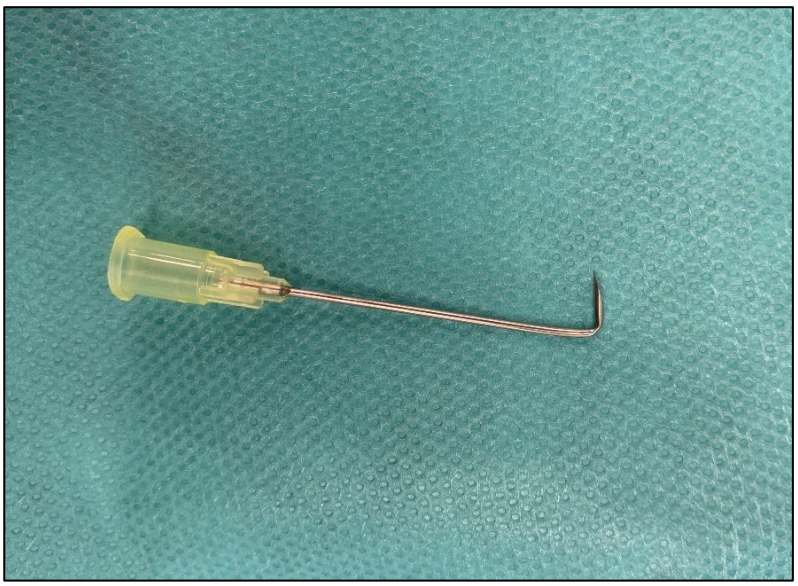


Figure 5: *Modified size G-20 needle for puncturing the thoracic duct.*

The catheter

In addition to the basic instruments, a transparent polyethylene tube of 0.40 mm inner diameter (ID) and 0.80 mm outer diameter (OD) is used (**Figure 6**).

The end puncturing the duct is cut with scissors, to form a beveled point. In preparation to facilitate handling, this end is steeped in 100 °C water, which makes reshaping and fixing into an “L-shaped loop” possible. To maintain the free flow of lymph fluid and avoid the coagulation of the lymph fluid in the catheter, it is important to flush the catheter with heparinized saline.

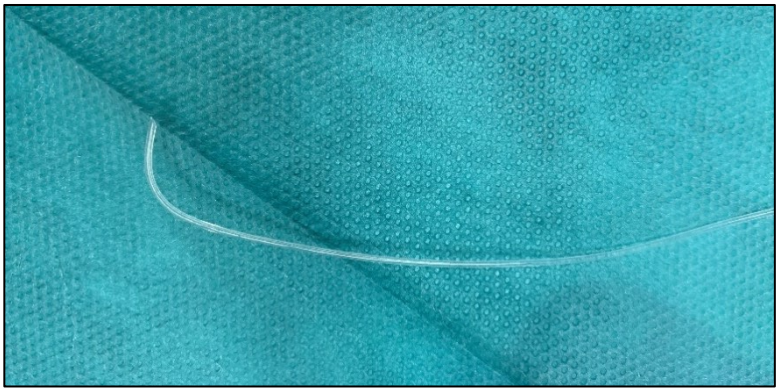


Figure 6: *Modified polyethylene catheter with an L-shaped, beveled end.*

4.2 Analgesia and anesthesia

For pain relief the animals were given 100 mg/kg Metamizole, Metamizol WDT subcutaneously half an hour prior to surgery. Analgesia is maintained with a second injection 4 hours later. The induction of anesthesia was achieved with a 1:1 oxygen flow containing 8 % Sevoflurane, Sevorane® in a chamber. To maintain the anesthesia during surgery, the rat was transferred to a nose cone and a 4.5 % Sevoflurane solution, Sevorane® with a 1:1 oxygen flow. The Sevoflurane solution, Sevorane® level was adapted during the procedure depending on the individual animal’s surgical state.

4.3 Preparation of rats for surgery

Before surgery, the rats were weighed and their general health was assessed by a general veterinary examination. After induction of anesthesia, the fur was shaved caudally of the *manubrium sterni* to the femoral area.

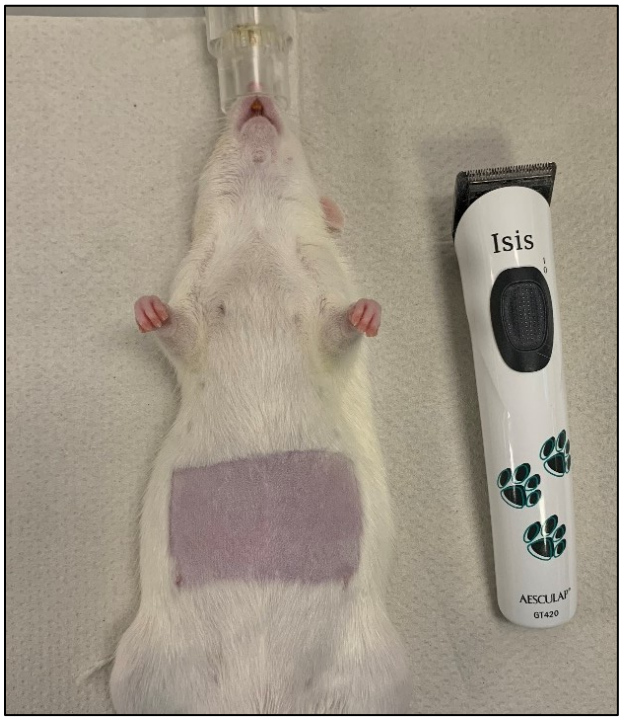


Figure 7: *Shaved anesthetized rat.*

Three different disinfectants were used and each of them was applied three times in concentric circle pattern (Braunol®, B. Braun, Braunoderm®, B. Braun, Kodan®, Schülke). Then, the animal was positioned underneath the microscope on the Heated Surgical Platform unit of the Rodent Surgical Monitor+, Indus Instruments to prevent hypothermia. The temperature of the animal was controlled and maintained throughout the entirety of the procedure by Temperature probe unit of the Rodent Surgical Monitor+, Indus Instruments, which was connected with the 9.7" Touchscreen Display Unit of the Rodent Surgical Monitor+, Indus Instruments. The rat was placed in dorsal recumbency, pointing with the head in the direction of the investigator.

4.4 The general surgical procedure

For opening the abdominal cavity, a transverse incision was made inferior of the xiphoid, caudal to the last ribs. With help of the magnetic fixator retraction system, skin and muscles were retracted widely.

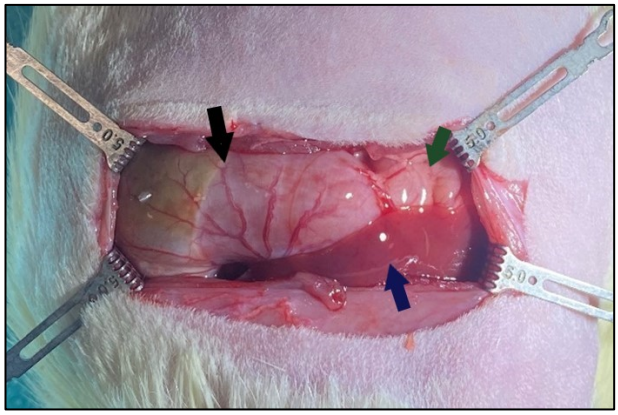


Figure 8: *Opened abdominal cavity with protruding organs (stomach (black arrow), liver (blue arrow) and intestines with omentum (green arrow)).*

The left kidney and adrenal gland were separated from the connective tissue and fat by gently pulling with cotton tips. The non-woven gauze, moistened with warm sodium chloride solution, was used to push the kidney and intestines caudally and the liver together with the stomach and spleen to the left side of the animal. This prevented the organs from drying out and helped to keep them in place for securing a clear operative field.

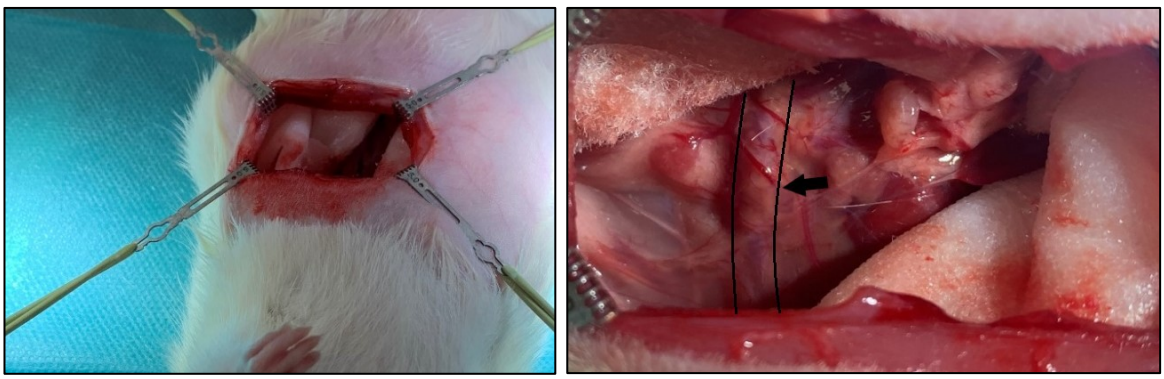


Figure 9 *(left)****:*** *Free surgical field and* ***Figure 10*** *(right): Free surgical field with pulsating abdominal aorta (black arrow).*

Due to its pulsation, the abdominal aorta was now easily identifiable. The thoracic duct was lying parallel and partially dorsally of the aorta. The aorta and the lymph duct were separated by removing the surrounding connective tissue. For separating these two structures, the forceps needed to be spread several times very gently. Because of the more resilient wall of the aorta, it was important to direct the points of the forceps towards the aorta. It was imperative not to touch the thoracic duct during this procedure as it would easily break. Furthermore, the vertebral arteries crossing over the duct hampered the exposure. Even

though these vessels are very small, a rupture could cause bleedings, which complicated the critical part of the surgery by obscuring the field of vision.

Then, using the forceps, the lymph duct was separated from the left psoas muscles. Once the connective tissue was removed completely and the duct was exposed, the formed gap between the aorta and thoracic duct was used, to pass a black 6/0 silk ligature around the duct. The thread is positioned as cranial as possible and is tied down tightly with a surgical knot. The lymph vessel immediately distends to about a similar size of the aorta. A second silk ligature was looped around 8 mm caudally of the first one and was left loosely around the duct (**Figure 11**).

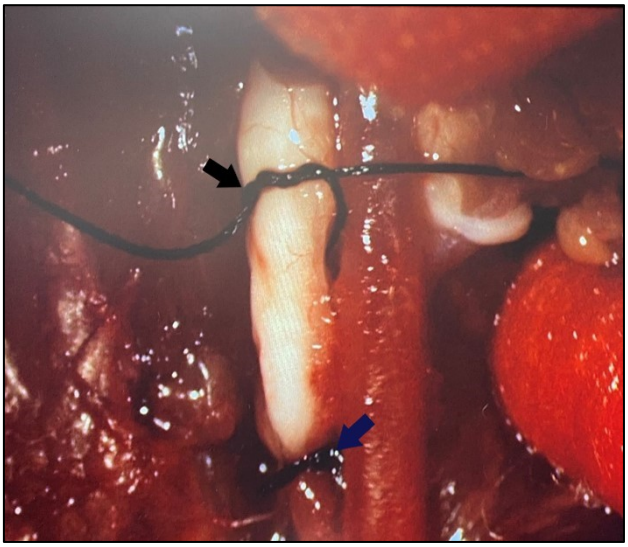


***Figure 11**: Thoracic duct with ligature (blue arrow) and loop (black arrow) in place.*

The size G-18 needle was pierced through the left lateral abdominal wall, at the level of the punctation site. The polyethylene catheter, flushed with heparinized saline, was passed through this needle and situated with the "L-shaped" end positioned along the *ductus thoracicus*. The needle was removed, leaving the tube in place.

The tip of the size G-20 needle was bent in a 90 ° angle, with the sharp end outside (**Figure 6**). With this L-shaped needle the lymph vessel was punctured caudally to the prepared first ligature and the polyethene catheter was introduced through the hole made by the needle into the duct. Immediately, the milky lymph fluid appeared inside the transparent tube. It is impossible to prevent that some of the lymph flows out through the hole next to the catheter, so it was important to quickly close the caudally placed silk loop with a tight surgical knot.

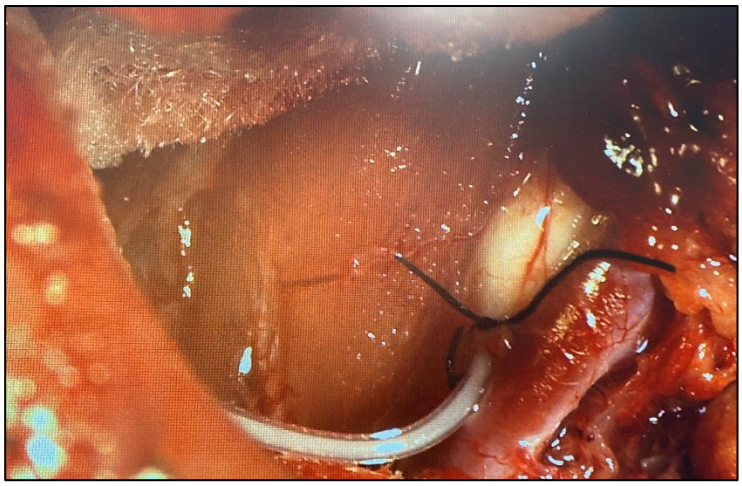


***Figure 12**: The cannulated thoracic duct.*

To secure the ligature and achieve a proper sealing, one drop of surgical glue was applied on the threads. The free end of the catheter led into a heparin tube, which collected the outcoming lymph. After removing the non-woven gauze, the intestines were replaced in their normal position. The organs and abdominal cavity were flushed with warm saline to moisten them.
For closing the muscle layer sultan stiches were placed, using a 4/0 Vicryl tapper needle. Using the single interrupted suture technique, the skin was closed with a 4/0 Vicryl cutting needle.

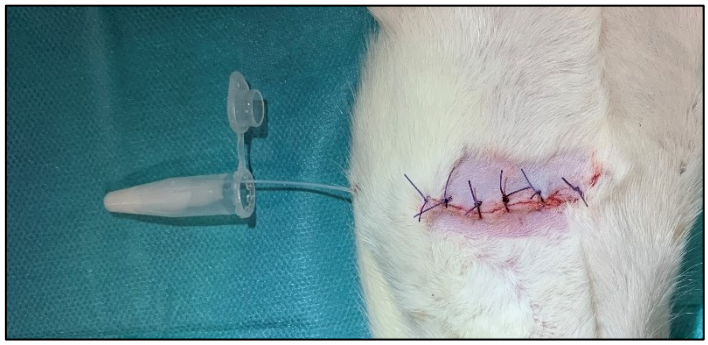


***Figure 13**: Lymph collection in a rat after the surgical procedure.*

4.5 Establishment of the surgical method – compound 1

In order to confirm the successful establishment of the surgical method for cannulating the thoracic duct, one male rat with a weight of 279 g was operated and received Vitamin D_3. The compound was dissolved in olive oil and administered via an oral gavage right after the cannulation of the lymphatic system. The selected dose of 0.5 mg/kg was described in the literature (Dahan & Hoffman, 2005). Time points of lymph collection time frame were after 30 minutes and 1, 2, 3, 4 and 5 hours after compound administration. Lymph fluid was collected in Eppendorf Safe-Lock Tubes, 1.5 mL. Sample storage and analysis was performed identically to the formulation experiments described in **Chapter 6.4**. The compound used for this experiment was ordered at Sigma-Aldrich® company under the product name Cholecalciferol

≥ 98 % (HPLC), 1 g (product number: C9756). The substance was stored light protected, at 4 °C, until its administration.

5 The compound and formulation preparation

5.1 Compound 1

To confirm the establishment of the surgical method of the cannulation of the lymphatic system compound 1 was administered orally. For this, Cholecalciferol ≥ 95 % (HPLC), C9756-1G, Sigma Aldrich, a white powder, stored at 4 °C, was used. Freshly before dosing 0.5 mg of Vitamin D_3 were dissolved in 2 mL of olive oil for oral administration.

5.2 Compound 2 and 3

Both formulations, respectively meet the criteria of lipid based and non-lipid based. They were internally used standard formulations in toxicology and formulation studies and known to be well tolerated. Compounds 2 and 3 were provided internally by AbbVie.

Materials needed:

- Dimethyl sulfoxide (DMSO)
- Polyethylene glycol 400 (PEG400)
- Cremophor EL
- Oleic acid

Equipment needed:

- Pipettes (in different sizes depending on target formulation volume)
- Vortexer (IKA Vortex 3 Schüttler)
- Scale e.g. “Analysewaage, Mettler Toledo” up to 52 g

5.2.1 Preparation of vehicles

a. *Non-lipid: 10 % DMSO: 90 % PEG400*
   Both components were pipetted together and vortexed until the mixture was homogenous (vehicle 1).

b. *Lipid: 10 % PEG400: 10 % Cremophor EL: 80 % Oleic acid*
   All components were pipetted together and vortexed until the mixture was homogenous (vehicle 2).

5.2.2 Calculations

Vehicles

The vehicles were prepared volumetrically (v/v %) i.e. for 100 mL of vehicle 1 (non-lipid) with 10 % DMSO and 90 % PEG400 the following volumes were pipetted:

- 10 mL DMSO
- 90 mL PEG400

Active pharmaceutical ingredients (API) amount

The amount of API was calculated based on the target volume, the purity and salt factor of the compound as well as the target concentrations as follows:

- $m_{(API)}$ [mg] = concentration [mg/mL] * volume [mL] * salt factor * (100/purity [%])

Formulations

The amount of vehicle was calculated based on the API weighing. Since the formulation resulted in a solution, the API density could be neglected:

- $volume_{(vehicle)}$ [mL] = $m_{(API)}$ [mg] / concentration [mg/mL]

5.2.3 Storage and stability

Compound 2

Both formulations were prepared on day 1, 5 and day 10. They were stored at 4 °C and light protected. Before use they were warmed up to room temperature for a minimum of 15 minutes before administration.

Stability report of the non-lipid and lipid based formulation

Stability was tested and the compound solution showed sufficient physical stability after 7 days when stored at 4 °C and light protected in a concentration of 6 mg/mL in both vehicles (**Appendix, Section 3.1**).

Compound 3

Following the stability report the lipid based formulation was newly prepared weekly, while the non-lipid formulation was only prepared on the first day and the beginning of the third week of the experiment. Both formulations were stored protected from light at 4 °C and ultrasonicated for 45 minutes before use.

Stability report of the non-lipid formulation

The compound solution showed sufficient physical stability after 14 days stored at room temperature or at 4 °C. It also showed sufficient chemical stability after 14 days stored at room temperature or at 4 °C, light protected (**Appendix, Section 3.2**).

Stability report of the lipid based formulation

The compound solution had a sufficient physical stability after 24 hours stored at room temperature. It showed sufficient chemical stability after 4 days stored at room temperature and after 14 days stored at 4 °C, light protected.
From day 2 on, the lipid formulation needed to be ultrasonicated (up to 45 min) in order to redissolve the API (**Appendix, Section 3.2**).

5.3 Compound 4

For experiments conducted with compound 4, Purified Mouse Anti-Rat α/β T-Cell Receptor, Clone R73, BD Biosciences was used. The antibody was delivered in an aqueous buffered solution containing ≤ 0.09 % sodium azide, in a concentration of 0.5 mg/mL and stored undiluted at 4 °C. Approximately ½ hour before subcutaneous administration the compound was taken out of the fridge to bring it to room temperature.

6 Sample collection, storage and analysis

For formulation experiments

6.1 Lymph and blood sampling analysis

The lymph samples were always obtained over a collection period in animals which had undergone surgery. The baseline values were collected after successful cannulation of the thoracic duct until the formulation was administered. Further time points of the collection time frame were 15, 30 minutes and 1, 2, 3, 4, 5 and 6 hours after compound administration. Lymphatic fluid was collected in 1.3 mL Lithium Heparin, Plasma Micro tubes and placed on ice. For storage at -20 °C until the subsequent concentration analysis, the lymph was pipetted into a Micronic 1.10 mL 2D Data-Matrix coded Screw Cap tubes V-bottom (**Figure 14**). Blood samples were taken at the appropriate time points, baselines, 15, 30 minutes and 1, 2, 3, 4, 5 and 6 hours after compound administration. For determination of drug levels, 120 µL/blood samples were collected in potassium Ethylenediaminetetraacetic acid (EDTA) (Microvette® CB300 K2E), SARSTEDT AG & Co. KG and centrifuged in a centrifuge set at 4000 rpm and 4 °C. The plasma was pipetted in Micronic 1.10 mL 2D Data-Matrix coded Screw Cap tubes V-bottom stored -20 °C until test item concentrations were analyzed.

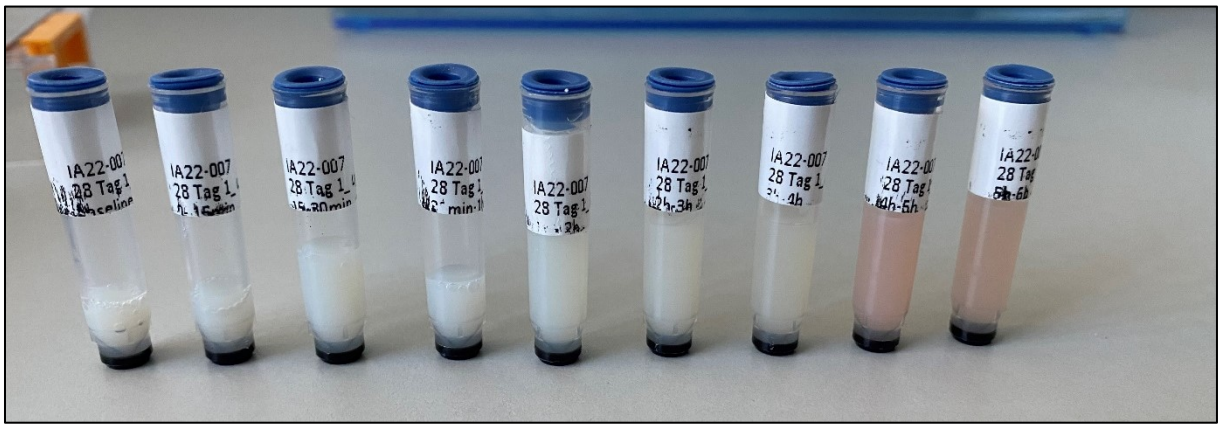


***Figure 14**: Collected lymph fluid at baseline and time frame time points 15, 30 minutes and 1, 2, 3, 4, 5 and 6 hours after compound administration in Micronic 1.10 mL 2D Data-Matrix coded Screw Cap tubes V-bottom.*

6.2 Technical methods

Plasma and Lymph samples were frozen at -20 °C. After finalization of the last experiment day, they were submitted to the DMPK Department for the analytic process. Sample preparation was performed, using the Hamilton Microlab Star M Robot and the sample analytic using the SCIEX Triple Quad™ 6500+ LC-MS/MS System, Sciex.

6.3 Lymph and plasma sample preparation

Standard preparation

Intermediate is prepared from compound stock solution using sterile water. Calibrators S1 to S11 were prepared from intermediate using blank plasma, 5 times diluted in water. 50 µL of each calibrator was transferred and 400 µL of Internal Standard dissolved in acetonitrile was added.

Sample preparation

40 µL of blank water was added to 10 µL of plasma sample. Then, 400 µL of Internal Standard dissolved in acetonitrile was added.

6.4 Lymph preparation

Standard preparation

Intermediate was prepared from compound stock solution (compound in dimethylformamide (DMF) at 0.1mg/mL) using water. Calibrators S1 to S11 were prepared as serial dilution from intermediate using blank plasma. 50 µL of each calibrator was transferred into 50 µL blank lymph fluid and 400 µL of Internal Standard dissolved in acetonitrile was added.

Lymph preparation

40 µL of blank water was added to 10 µL of lymph sample. Then, 400 µL of Internal Standard dissolved in acetonitrile was added.

6.5 Processing

After the preparation steps, microplates were tightly closed and shaken. Subsequently, microplates were centrifuged for 10 minutes at 3000 g and 4 °C. 125 µL of supernatant was transferred into a microplate and 125 µL of 0.1 % formic acid in water was added.

6.6 Analysis

The compound and spiked internal standard Global Internal Standard (GIS) were separated from sample (plasma/lymph) matrix by protein precipitation using acetonitrile. Chromatography of the extracted sample supernatant was conducted using a Waters HSS T3 50 * 2.1 mm 1.8 µm column running a mobile phase gradient with a constant flow rate of

0.7 mL/min (mobile phase A: 0.1 % formic acid in water, mobile phase B: 0.1 % formic acid in acetonitrile).

Detection was performed on a SCIEX Triple Quad™ 6500+LC-MS/MS System, Sciex using electro spray ionization. Analyte and internal standard peak areas were determined using software 'Sciex Analyst version 1.7.2'. The concentration of each sample was calculated by least squares regression analysis of the analyte / internal standard ratio, using a calibration curve spiked with defined concentration analyte in equivalent matrix.

6.7 Statistical methods

6.7.1 Determination of group size number

To confirm the successful establishment of the surgical method and to generate data as a planning basis for the main study, a pilot study was carried out with an in-house compound. The obtained information was considered for the study design and determination of the number and size of groups for the following studies. Lymphatic and plasma concentration data of all groups were utilized for statistical analysis.

6.7.2 Data analysis

For formulation experiments

Since concentration levels at 15 and 30 minutes were not measurable in the majority of animals, concentrations at time points 1 hour till 6 hours were considered as appropriate for all subsequent statistical analyses.

First, data were analyzed using repeated measured two-way Analysis of Variance (ANOVA). Analyses were done presuming normal or log-normal underlying distributions. To explore the underlying distribution of the data, Shapiro-Wilk's tests were performed to assess a normal distribution. If needed, data were transformed onto a logarithmic scale and the homogeneity of variances was assessed using Levene's test.

Additional analysis based on Area Under the Curve (AUC) values calculated in all groups on individual animal level were carried out to assess the overall behavior of concentration data for each animal. Group comparisons were done by 2 samples t-test assuming normal or log-normal distribution and homogeneity of variances. The underlying distribution and variability were assessed using Shapiro-Wilk's test and Levene's test, as described above.

Statistical analyses were carried out with JMP 14.1.0 (SAS institute) Software and R studio Version 1.1.453. Exact p-values are reported in the tables. Differences were considered to be significant at $p \leq 0.05$.

For subcutaneous experiments

Cell compositions at all time points were considered as appropriate for all subsequent statistical analyses. First, data were evaluated by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum. Next, a paired t-test was conducted in group 2, to assess the difference between change in cell population in baseline lymph and baseline blood samples at later time points. Groups comparison was done utilizing an unpaired t-test.

Statistical analyses were carried out with GraphPad Prism 9.1.0 Software (GraphPad software, San Diego, CA). Tests were considered to be significant at $p \leq 0.05$.

6.8 Lymph smear preparation

In order to analyze the cellular composition of the lymph, freshly collected lymph fluid was used to prepare a lymph smear. It was stained with 'Wright Staining' (Rinse concentrate A (buffer) pH 6.8, reagent B with thiazine, reagent C with eosin and methanol), using the Aerospray Stainer and analyzed by light microscopy.

6.9 FACS analysis

Venous blood was collected from sublingual vein in 500 µL K_2EDTA 1.0 mg Microtainer, BD Biosciences and stored at 7 °C. After having finished the last blood collection all samples were handed over for the FACS preparation and analysis, carried out the same day. Blood collection time points were at baseline, 15, 30 minutes and 1, 2, 3, 4 and 24 hours after compound administration. The lymph samples were always obtained over a collection period in animals which had undergone surgery. After successful cannulation the baseline values were collected in 1.3 mL Lithium Heparin, Plasma Micro tubes until 1 mg/kg of compound 4 was administered. Further time points of the collection time frame were 15, 30 minutes and 1, 2, and 3 hours after compound administration. The tubes were placed in an Isosafe 0 °C Isopack, Eppendorf for keeping the samples cooled. After each collection time frame samples were stored at 4 °C till the last collection time point. In addition baseline and terminal blood collection was performed in operated animals. Blood samples were collected in 500 µL K2EDTA 1.0 mg Microtainer, BD Biosciences. The same day, after the last collection time point, all samples were sent to the Analytical Platform, PCS unit for further processing and analysis.

6.9.1 Immunophenotyping

Lymph and blood samples were prepared according to the "RAT EDTA Blood Preparation" protocol (**Appendix, Section 4**). For defining the cell populations following staining pattern was used (**Table 7**). FACS analyses were carried out with BD FACSVerse™ Cell Analyzer, BD Biosciences.

Flow Cytometry Parameters	
Cell Population	**Phenotype**
T-cells	SSClo/med/CD45+/CD3+/CD45R-
T-helper Cells	SSClo/med/CD45+/CD3+/CD4+/CD8a-
Cytotoxic T-Cells	SSClo/med/CD45+/CD3+/ CD4-/CD8a+
B-cells	SSClo/med/CD45+/CD3-/CD45R+
NK[a] cells	SSClo/med/CD45+/CD3-/CD161ahigh
NK T-cells	SSClo/med/CD45+/CD3+/CD161a+

Table 7: *Immunophenotypic markers in specific T-cell types.*

a. Natural killer cells

7 Formulation experiments

7.1 Study plan

To ensure that the study processes were carried out as planned, a study plan was written for each study and all study details were communicated internally at a joint meeting with personnel involved in sample analysis, *in-vivo* support (blood sampling and dosing) and statistical evaluation. Changes made to the original study plan were announced under "Amendments". All study information was shared via e-mail and is stored internally on a shared drive and in print in a raw data folder.

The study plans describe the housing conditions according to the species, responsibilities (e.g. study director, deputy study director, lead technician, veterinarian, etc.) and timelines. They also provide details regarding the test item (formulation, storage conditions, and dose). Furthermore, the course of the study, the surgical procedures and data that must be recorded during the study (body weights, food intake, lymph and plasma samples for PK, etc.) have been defined. In addition to sample collection, the handling, storage and transport of the samples was also described in the study plan.

7.2 Compound 2

7.2.1 Dose selection

In this study, rats received oral single doses of 3 mg/kg/day compound 2 in 10 % DMSO: 90 % PEG400 and of compound 2 in 10 %PEG400: 10 % Cremophor EL: 80 % Oleic acid. This dose was selected because there are historical reference data for comparison. Previous toxicology rat studies with an oral dose of 10 mg/kg showed reversible, decreased circulating platelet and neutrophil counts, single-cell necrosis in liver, parotid salivary gland, seminal vesicles and ureters, increased mitotic index, hypertrophy or hyperplasia of Kupffer cells and pigmentation of Kupffer cells in liver. All of the aforesaid changes were reversible. Also the potential cardiovascular liabilities of compound 2 were tested in the anesthetized dog model and a decrease in mean arterial pressure (-21 mmHg) and cardiac output (-27 %) as well as an increase in heart rate (+23 beats/min) compared to baseline were observed at 7 mg/kg i.v. up to 150 minutes post-dose. Since the duration of the planned anesthesia in this study was longer, an oral single dose of 3 mg/kg was selected based on the overall historical data. Based on data from a DMPK study with oral dosing of 5 mg/kg compound 2 in 10 % DMSO: 90 % PEG400, the period of sample collection over 6 hours was selected.

7.2.2 Study design

The experiments were conducted over a period of 15 days. During the first 13 days, one terminal animal was operated per day for the cannulation of the lymphatic system and the

associated lymph collection. The experiments with the satellite groups were carried out on day 14 and 15. Altogether 37 male rats were ordered, 36 rats were used in the study, one animal was used as reserve animal. To ensure a similar weight of 200-350 g, animals were ordered separately for each study week.

Rats received an administration of 0.1 mL olive oil approximately 1 hour before starting the surgery. After successful cannulation, lymphatic fluid was collected for baseline analysis. Then a single dose of 3 mg/kg compound 2 in a non-lipid or lipid vehicle was administered directly into the stomach. For analyzing compound 2 concentrations, lymphatic fluid was collected for a period of 15 minutes after administration, 15-30 minutes and 30 minutes till 1 hour after administration. From 1-hour onwards, the collecting tube was changed every hour up to 6 hours after administration. During the entire time of lymph collection the animals were under anesthesia. They were euthanized at the end of experiment. Due to the surgery-related interruption of the systemic uptake via the lymph, additional satellite animals not canulated were dosed for plasma level measurements of compound 2.

To evaluate the influence of the olive oil that was administered before surgery, two satellite groups were dosed exclusively with the formulations while the remaining two received the olive oil and formulation. The time between oil and substance administration corresponded with the interval of the operated animals. Blood was collected from tail vein at baselines, 15, 30 minutes and 1, 2, 3, 4 ,5 and 6 hours after compound administration. During surgery the animal data, anesthesia related data, time of analgesia administration, time of infusion, time of oil administration, amount and time of substance administration, time of lymph collection and additional comments were documented in a surgery protocol (**Appendix, Section 2**). Food was removed 12 hours prior to the dosing day for the duration of dosing and sample collection period. For satellite rats, food was available again after the last blood collection time point. Animals undergoing surgery had water *ad libitum* accessible till the beginning of the anesthesia, satellite animals had water continuously available *ad libitum*. In order to compensate the fluid loss induced by the surgery and sample collection, saline, NaCl 0.9 %, Braun® was administered into the abdominal cavity of the animals every hour from the 2-hour value onwards.

Group 1	**Satellite group 1**	**Group 2**	**Satellite group 3**
Lymph collection under anesthesia, injection of compound 2 in vehicle 1 directly into the stomach	Satellite animals: Without surgery, oral administration of compound 2 in vehicle 1 via oral gavage after oil administration	Lymph collection under anesthesia, injection of compound 2 in vehicle 2 directly into the stomach	Satellite animals: Without surgery, oral administration of compound 2 in vehicle 2 via oral gavage after oil administration
	Satellite group 2		**Satellite group 4**
	Satellite animals: Without surgery, oral administration compound 2 in vehicle 1 via oral gavage without oil administration		Satellite animals: Without surgery, oral administration of compound 2 in vehicle 2 via oral gavage without oil administration

Table 8: *Group design.*

Test group	**Vehicle[a]**	**Dosage (mg/kg)[b]**	**Concentration (mg/mL)**	**Number of rats[c]**
				Male
1	vehicle 1	3	6	6 (6+6)
2	vehicle 2	3	6	6 (6+6)

Table 9: *Study design.*

a. Vehicle 1: 10 % DMSO: 90 % PEG400

Vehicle 2: 10 % PEG400:10 % Cremophor EL: 80 % Oleic acid

b. Dose volume 0.5 mL/kg

c. 6+6 male rats per group will be designated satellite rats used for determination of plasma test item concentrations.

7.3 Compound 3 – Itraconazole

7.3.1 Dose selection

Itraconazole is an already marketed and well tolerated drug that is used in both human and veterinary medicine for the treatment of fungal infections. In this study a single dose of 3 mg/kg Itraconazole was administered orally. The recommended initial oral dose in rats is 2.5 - 10 mg/kg. Observed side effects were described at doses of 40 - 160 mg/kg (hepatotoxicity, anorexia, increased adrenal weights and accumulation of proteinaceous material in the mononuclear phagocyte system, maternal toxicity, embryo toxicity, and teratogenicity). Since animals were also exposed to a long anesthesia in this experiment, the dose selection was based on the lower range of the clinical initial dose.

7.3.2 Study design

The study design was structured in the same way as the study design of compound 2. The formulations, sample collection time points, number of groups and group sizes were also identical. All important events during surgery were documented in a surgery protocol for each animal (**Appendix, Section 2**).

8 Immunology experiments

8.1 *In-vitro* pilot study

This *in-vitro* pilot study was carried out considering the 3R rules in order to analyze whether the antibody is suitable for the main experiment and to keep the number of animals as low as possible. The experiment was performed with blood samples from rats. Blood sample data were assessed to determine if the antibody showed the desired activating influence on the number of lymphocytes. Furthermore, the suitability of the antibody used for staining and the adjusted FACS panel was evaluated.

8.1.1 Dose selection

For the *in-vitro* experiment, the same dose was used as for the planned *in-vivo* study, described in **Chapter 8.2.** When administered subcutaneously, it is estimated that 60 % of the compound reach the blood circulation. Rats have about 60 mL blood/kg (Wolfensohn & Lloyd, 2008; Dülsner *et al.*, 2017). Calculated down to 1 mL of blood, the dosage was 0.01 mg/mL blood.

8.1.2 Study design

Four individual 500 µL K2EDTA 1.0 mg Microtainer, BD Biosciences each with 0.5 mL fresh rat blood were collected from the sublingual vein of a male anesthetized rat. The antibody was added directly to one sample, while a second sample was left untreated and used as a control sample. The two remaining samples were centrifuged at 200-300 G for 5 minutes. Afterwards, the serum was pipetted off and the same amount of Gibco® Dulbecco's Modified Eagle Medium (DMEM) high glucose medium, Thermo Fisher Scientific as the pipetted serum was added to the remaining erythrocytes and white blood cell mixture. In the next step the compound was mixed with one of these samples, while the other sample remained untreated as control sample. All samples were incubated for one hour at 37 °C, using the Thermomixer comfort, Eppendorf. After the incubation time the blood samples were submitted to the Analytical Platform, PCS unit for the cell preparation and immunophenotyping by FACS analysis, carried out with BD FACSVerse™ Cell Analyzer, BD Biosciences.

8.2 *In-vivo* pilo study, compound 4 – R73 monoclonal antibody (mAb)

8.2.1 Dose selection

Animals received a single dose of 1 mg/kg compound 4 subcutaneously injected in the lateral upper part of the left and right hind leg. This dose was selected because previous reports have shown the good tolerability and the desired cellular effect in plasma after administering a multiple dose of 1 mg/kg intraperitoneally daily (Inoki *et al.*, 2002). The application site was chosen because of the well-drained legs with lymph vessels (Tilney, 1970).

8.2.2 Study

Test group	Sample	Dosage (mg/kg)[a]	Concentration (mg/mL)	Number of rats
				Male
1	Plasma	1	0.5	6
2	Lymph	1	0.5	6

Table 10: *Study design compound 4.*

a. Dose volume 1 mL/kg

The experiment was carried out staggered over 4 days. On the first day, all animals of group 1 were administered subcutaneously with 1 mg/kg of the compound 4. Venous blood was collected from sublingual vein in 500 µL K2EDTA 1.0 mg Microtainer, BD Biosciences and stored at 7 °C. After having finished the last blood collection all samples were handed over for the FACS preparation and analysis, carried out the same day. Blood collection time points were at baseline, 15, 30 minutes and 1, 2, 3, 4 and 24 hours after compound administration. Based on the results from group 1, the lymph collection time frames for group 2 were determined. Experiments of group 2 were split over three days. Two animals were operated per day for the cannulation of the thoracic duct. After successful cannulation the baseline values were collected in 1.3 mL Lithium Heparin, Plasma Micro tubes, Eppendorf until 1 mg/kg of compound 4 was administered. Further time points of the collection time frame were 15, 30 minutes and 1, 2, and 3 hours after compound administration. The tubes were placed in an Isosafe 0 °C Isopack, Eppendorf for keeping the samples cooled. After every collection time frame samples were stored at 4 °C till the last collection time point. All samples were sent on the same day to the Analytical Platform, PCS unit, for further processing and analysis.

Altogether 13 male rats with a weight of 200-350 g were used for this experiment. Each group had six animals, one rat was deployed as reserve animal. Group 1 had *ad libitum* free access to food and water. For animals of group 2 both were offered till the beginning of the surgery.

VI. RESULTS

1 Establishment of the surgical method

The establishment of the surgical method of the cannulation of the lymphatic system was confirmed by the experiment with oral administration of 0.5 mg/kg compound 1 (vitamin D_3) followed by measurement of vitamin D_3 concentrations in the collected lymph at different time points. It was possible to collect a sufficient amount of lymph fluid over a period of 5 hours from rats under anesthesia (**Figure 15**). The visualization of the lymphatic system was possible with the administration of 0.1 mL olive oil approximately 1 hour before the beginning of the surgery procedure. The duration of the surgery was on average 25 minutes from induction of anesthesia and achievement of surgical tolerance till the beginning of lymph collection and closure of the abdominal cavity. The selected size and weight of the animals was considered suitable for the experiment, however, the administration of the compound via an oral gavage in the anesthetized animals turned out to be difficult. This was related to the animal's supine position and the face mask used to maintain anesthesia during surgery. Due to the positioning of the rat, it could not be ensured that the entire amount of the compound reached the stomach. Furthermore, the Eppendorf tubes proved to be unsuitable for collection time frames of 1 hour because the lymph fluid started to clot during the lymph collection.

Vitamin D_3 concentrations could already be measured within the first 15 minutes after administration. From the 2-hour time frame onwards, an increase in concentration was observed not reaching a C_{max} within 5 hours. The highest measured concentration of compound 1 was 520.7 ng/mL measured at the 5 hours value.

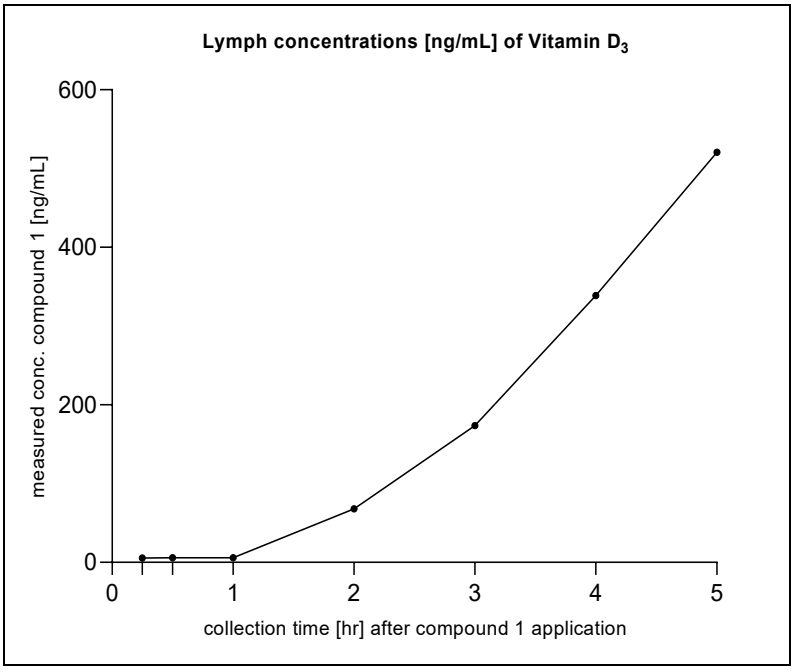


***Figure 15**: Mean concentrations of compound 1 in thoracic lymph fluid following oral administration in olive oil (n = 1) over 5 hours after compound administration.*

2 Formulation experiments

Raw data from concentration analysis (SCIEX Triple Quad™ 6500+ LC-MS/MS System) were summarized in an Excel Sheet by the Department of DMPK, AbbVie Deutschland GmbH & Co. KG, Ludwigshafen and submitted to the Data and Statistical Science (DSS) Department at AbbVie Deutschland GmbH Co. & KG, Ludwigshafen. Since concentration levels at 15 and 30 minutes were not measurable in the majority of animals, concentrations at time points 1 hour till 6 hours were considered as appropriate for all subsequent analyses.

In all experiments with compound 2 and compound 3, rats were assigned to different groups (six animals per group) to assess the drug kinetics in lymph and plasma under different conditions:

	Group 1	**Satellite group 1**	**Satellite group 2**	**Group 2**	**Satellite group 3**	**Satellite group 4**
Formulation based on	non-lipid	non-lipid	non-lipid	lipid	lipid	lipid
Oil-preadministration	X	X		X	X	
Lymph collection	X			X		
Plasma collection		X	X		X	X

Table 11: *Group and study design of formulation experiments.*

2.1 Compound 2 – Bcl-2 inhibitor

Comparison of lymph kinetics in non-lipid and lipid based formulation (group 1 and group 2)

A total number of 13 male rats were operated to collect lymphatic fluid. For analysis, the lymph samples from 10 animals were available. Because of intermittent lymph flow stop due to technical problems including coagulation of lymph fluid in the catheter, the samples of 3 animals could not be considered. In animals with an uninterrupted lymph flow the amount of collected lymph fluid ranged from 0.5 to 1.2 mL/h**. Figure 16** shows the mean lymph concentration-time profile over a period of 6 hours in thoracic duct-cannulated rats after an oral single dose administration of 3 mg/kg compound 2, either in a lipid based or non-lipid based formulation. The first concentrations could be measured after 15 minutes in the group of lipid based formulation, in contrast to the first measurable concentrations in the non-lipid

based formulation group after 30 minutes. From the 1-hour time point onwards a linear increase in concentration was observed in the lipid based group, not reaching a peak within 6 hours. The curve of the non-lipid formulation group remained constantly low with only a small increase. The maximum plasma concentration (C_{max}) after 2 hours in a male rat treated with the lipid based formulation was with 2100 ng/mL more than two fold higher compared to the highest measured concentration with 1082 ng/mL in an animal of the group administered with the non-lipid based formulation. The peak rates of mean measured lymph concentrations of compound 2 in animals orally dosed with lipid based formulation and non-lipid based formulation were 4626.2 and 723 ng/mL, respectively, indicating a greater than 6-fold increase in the maximum rate of lymphatic drug absorption in the presence of a lipid vehicle.

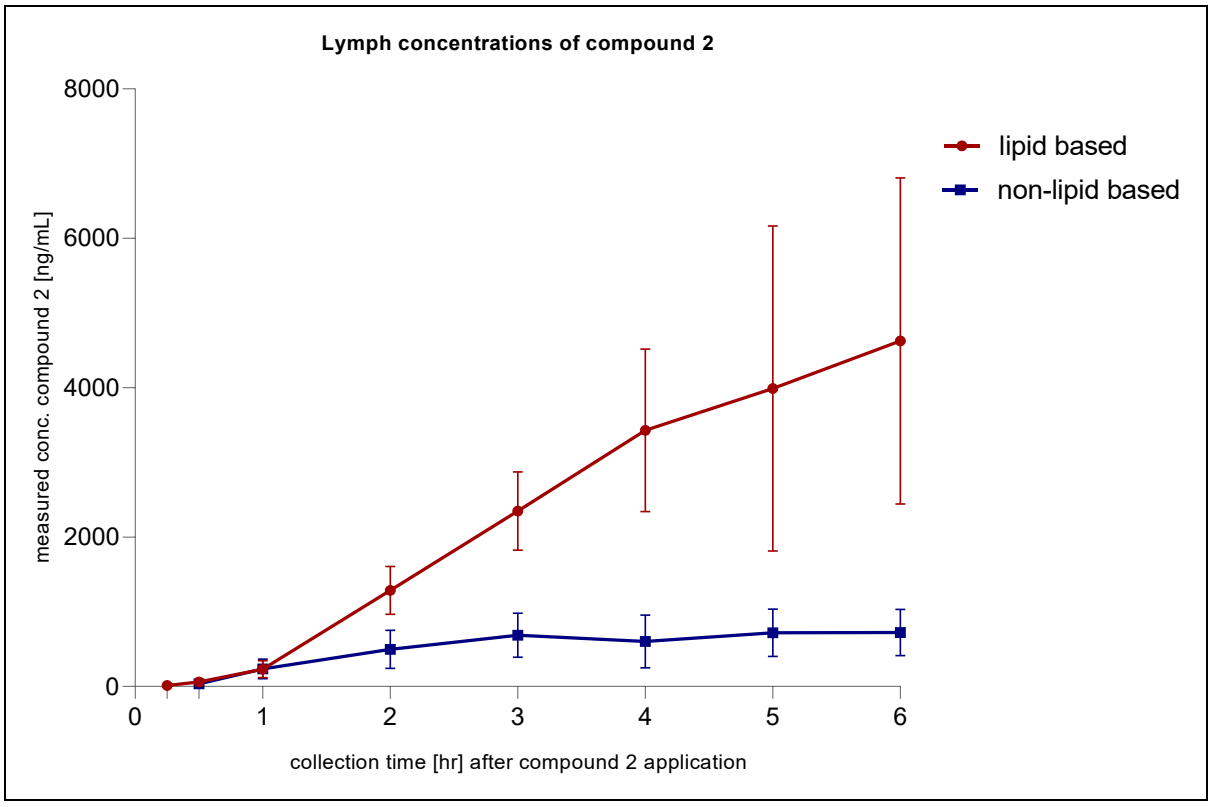


***Figure 16**: Mean concentration of compound 2 in thoracic lymph fluid following oral administration with either a lipid based formulation (n = 5, red) or non-lipid based formulation (n = 5, blue) over 6 hours after compound administration.*

The concentrations in the lymph of compound 2 in all animals of the lipid based group and the non-lipid based group were compared at each single collection time point. The data were evaluated as log transformed to fulfill the assumption of homogeneity. While the concentrations of the two groups did not differ significantly during the first 3 hours, the increase of concentrations in the lipid based formulation group was significantly higher at the time points 4, 5 and 6 hours, with $p < 0.05$ (**Table 12**).

Time point	SS[a]	DFNum[b]	DFden[c]	F Ratio	Prob > F
1 hr	3811	1	54	0.0009	0.9741
2 hrs	5e+5	1	54	0.1253	0.6177
3 hrs	4e+6	1	54	0.9566	0.0555
4 hrs	1e+7	1	54	3.3224	0.0473*
5 hrs	2e+7	1	54	5.6068	0.0142*
6 hrs	3e+7	1	54	7.3058	0.0054*

Table 12: *Statistical evaluation of concentrations of compound 2 in lymph, using repeated measures ANOVA , for time points 1 - 6 hours (*represents p ≤ 0.05).*

a. SS: Sum of squares
b. DFNum: Number of degrees of freedom
c. DFden: Degrees of freedom denominator

Comparison of plasma kinetics in non-lipid and lipid based formulation (satellite group 1 and satellite group 3)

The plasma concentrations of compound 2 were measured in blood samples collected before dosing (baseline) at 15 and 30 minutes and at 1, 2, 3, 4, 5, and 6 hours after 3 mg/kg oral compound 2 administration. In total, 12 satellite animals were used, without undergoing the surgical procedure but with pre-administration of olive oil similar to operated rats in order to assess the influence of the olive oil. Six animals of satellite group 1 then received compound 2 in the non-lipid based formulation and six animals of satellite group 3 received compound 2 in the lipid based formulation. The time between oil and substance administration corresponded with the interval of the operated animals. Plasma concentrations were measured in both groups and illustrated in **Figure 17**. Similar to the lymph concentrations in operated animals in groups 1 and 2, concentrations of compound 2 appeared earlier in the lipid based formulation compared to the non-lipid based formulation group in plasma. In all animals of satellite group 1 first concentrations were analyzed after one hour, while in satellite animals group 3 first concentrations were already measured after 30 minutes in all animals. In contrast to the lymph concentrations in groups 1 and 2, a peak could be determined in the plasma concentration curves for compound 2. The peak in the lipid based formulation group with a maximum mean concentration of 1151.7 ng/mL and the peak of the non-lipid based formulation group with a maximum mean concentration of 832.5 ng/mL were both reached at 3 hours.

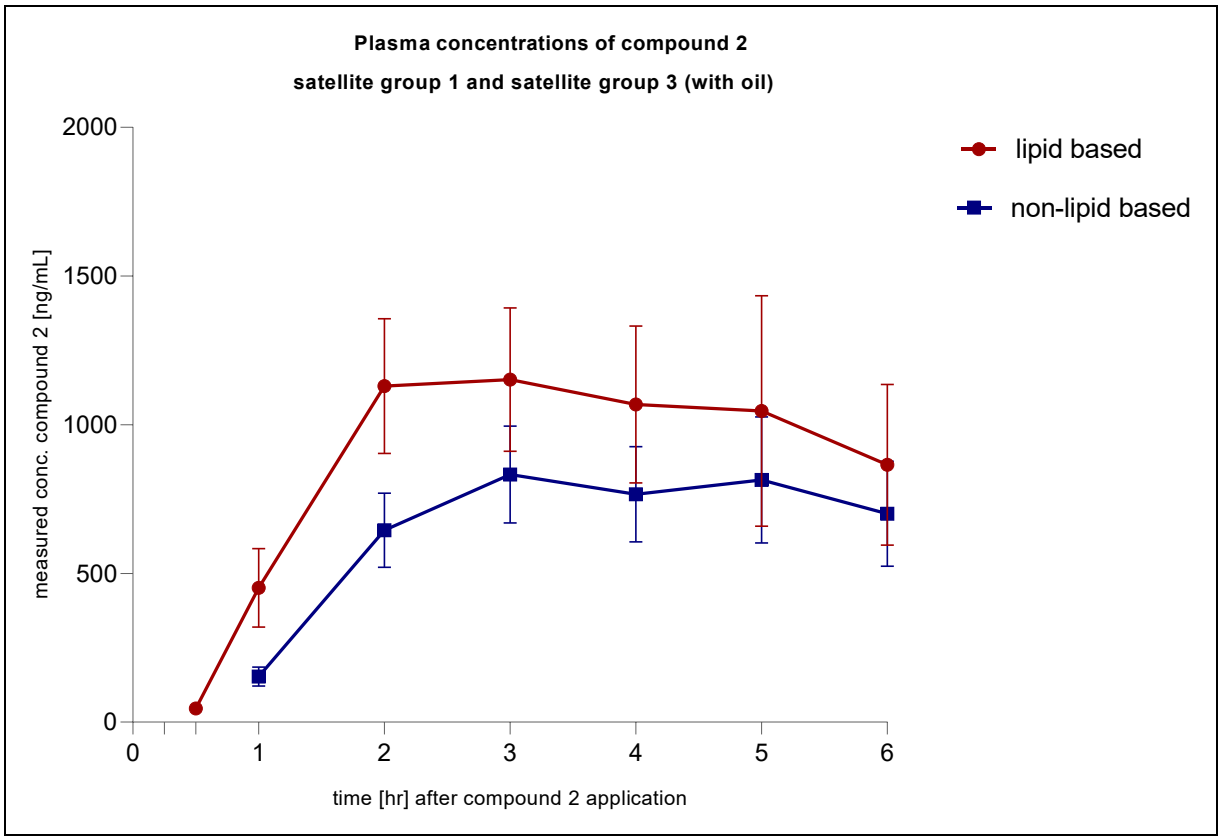


Figure 17: *Mean concentration of compound 2 in plasma of satellite animals group 1 and satellite animals group 3 following oral olive oil administration and oral administration with either a lipid based formulation (n = 6, red) or non-lipid based formulation (n = 6, blue) over 6 hours after compound administration.*

Starting from hour 2, an approximation of the two curves can be observed, which becomes more evident from hour 3 onwards. For comparison the plasma concentration data of compound 2 of both groups were log transformed. Except for hour 1 concentrations, there was no statistically significant difference between the mean plasma concentrations of the two groups (**Table 13**).

Time point	SS[a]	DFNum[b]	DFden[c]	F Ratio	Prob > F
1 hr	0.641	1	70	8.7368	0.0042*
2 hrs	0.168	1	70	2.2962	0.1342
3 hrs	0.052	1	70	0.7087	0.4027
4 hrs	0.051	1	70	0.6950	0.4073
5 hrs	0.022	1	70	0.3029	0.5838
6 hrs	0.025	1	70	0.3360	0.5640

Table 13*: Statistical evaluation of concentrations of compound 2 in plasma of satellite animals groups 1 and satellite animals group 3, using repeated measures ANOVA, for time points 1 - 6 hours (*represents p < 0.05).*

a. SS: Sum of squares
b. DFNum: Number of degrees of freedom
c. Dfden: Degrees of freedom denominator

Comparison of the influence of formulation and oil-preadministration (satellite group 2 and satellite group 4)

To evaluate the influence of the olive oil administered before surgery, satellite group 2 and satellite group 4 animals were dosed with 3 mg/kg compound 2 orally in lipid based vehicle 1 or non-lipid based vehicle 2 without oil-preadministration. The mean plasma concentration over a time course of 6 hours is illustrated in **Figure 18**.

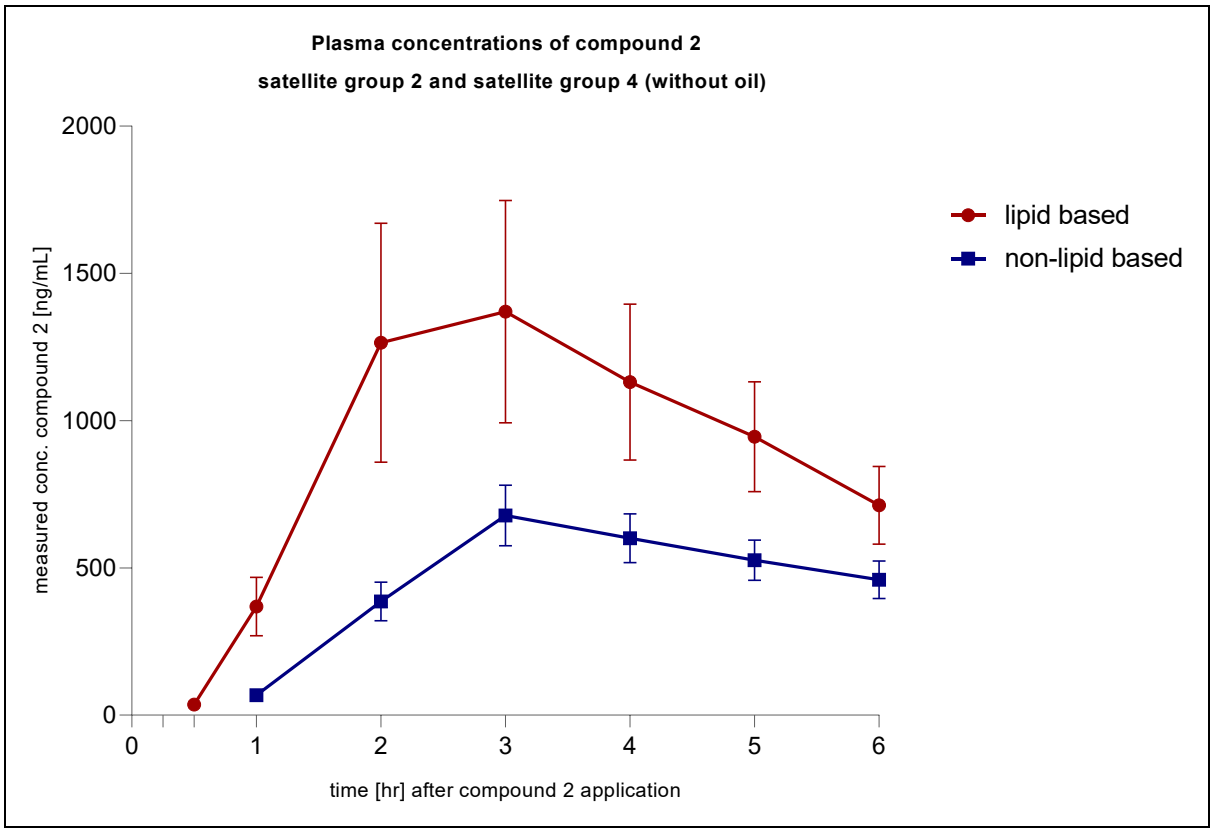


***Figure 18**: Mean plasma concentration-time profiles of compound 2 following oral adminitraion either in a lipid based formulation (n = 6, red) or non-lipid based formulation (n = 6, blue) over 6 hours after compound administration.*

As in the other groups described above, the first concentrations in the lipid based formulation group could already be measured after 30 minutes, while the first concentrations in the non-lipid formulation group were determined after one hour. Both curves show a similar course, each with a time of maximal plasma concentration (T_{max}) at 3 hours. The lipid based formulation showed a two-fold increase in compound 2 plasma concentrations as compared with non-lipid formulation. The C_{max} of satellite animal group 2 was 677.7 ng/mL while the C_{max} of satellite animal group 4 was 1370 ng/mL.

During the entire time course no overlap of the maximum plasma concentrations of the non-lipid formulation group and the minimum concentrations of the lipid formulation group could be observed. During the first 4 hours log transformed data showed a statistically significant difference between the two formulation groups (**Table 14**).

Time point	SS[a]	DFNum[b]	DFden[c]	F Ratio	Prob > F
1 hr	1.681	1	62	23.5660	<.0001*
2 hrs	0.574	1	62	8.0521	0.0061*
3 hrs	1e+6	1	62	6.2958	0.0147*
4 hrs	8e+5	1	62	4.6994	0.0490*
5 hrs	5e+5	1	62	3.2083	0.0538
6 hrs	2e+5	1	62	0.8449	0.3616

Table 14: *Statistical evaluation of concentrations of compound 2 in plasma of satellite animals group 2, using repeated measures ANOVA, for time points 1 - 6 hours (*represents p ≤ 0.05).*

a. SS: Sum of squares
b. DFNum: Number of degrees of freedom
c. DFden: Degrees of freedom denominator

Olive oil influence

In order to show the influence of the oil administration on exposure, the plasma measurements of the two non-lipid based formulation groups of the satellite groups with or without oil pre-administration were compared with each other (**Figure 19**). When comparing the mean plasma concentration data at each time point, no significant difference could be determined between the groups. Also, the two lipid based formulation groups show no significant difference in concentrations. The group that received olive oil before the administration reached a C_{max} of 1151.7 ng/mL, while the group which received the lipid based formulation had a C_{max} of 1370 ng/mL. For both groups T_{max} was after 3 hours (**Figure 20**).

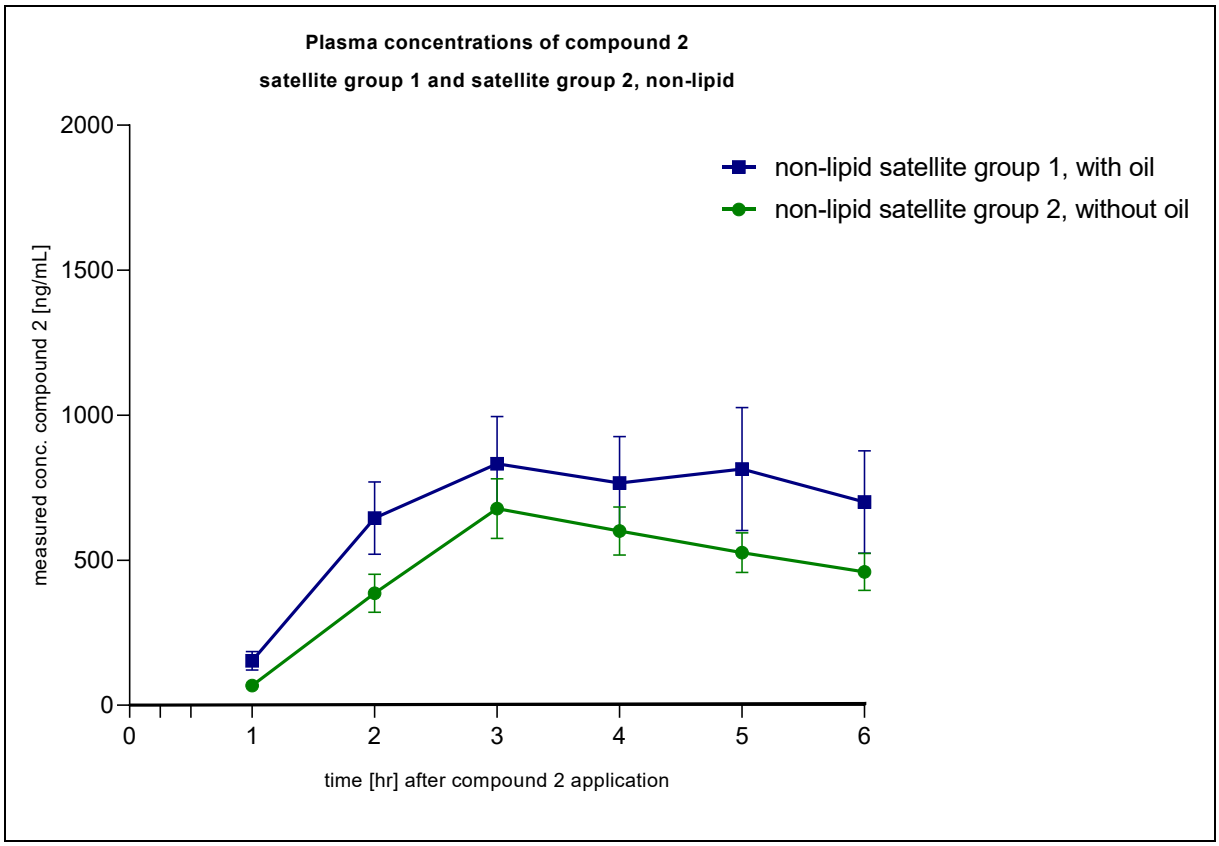


Figure 19: *Mean plasma concentration-time profile of compound 2 in satellite animal groups in non-lipid based formulation over 6 hours after administration.*

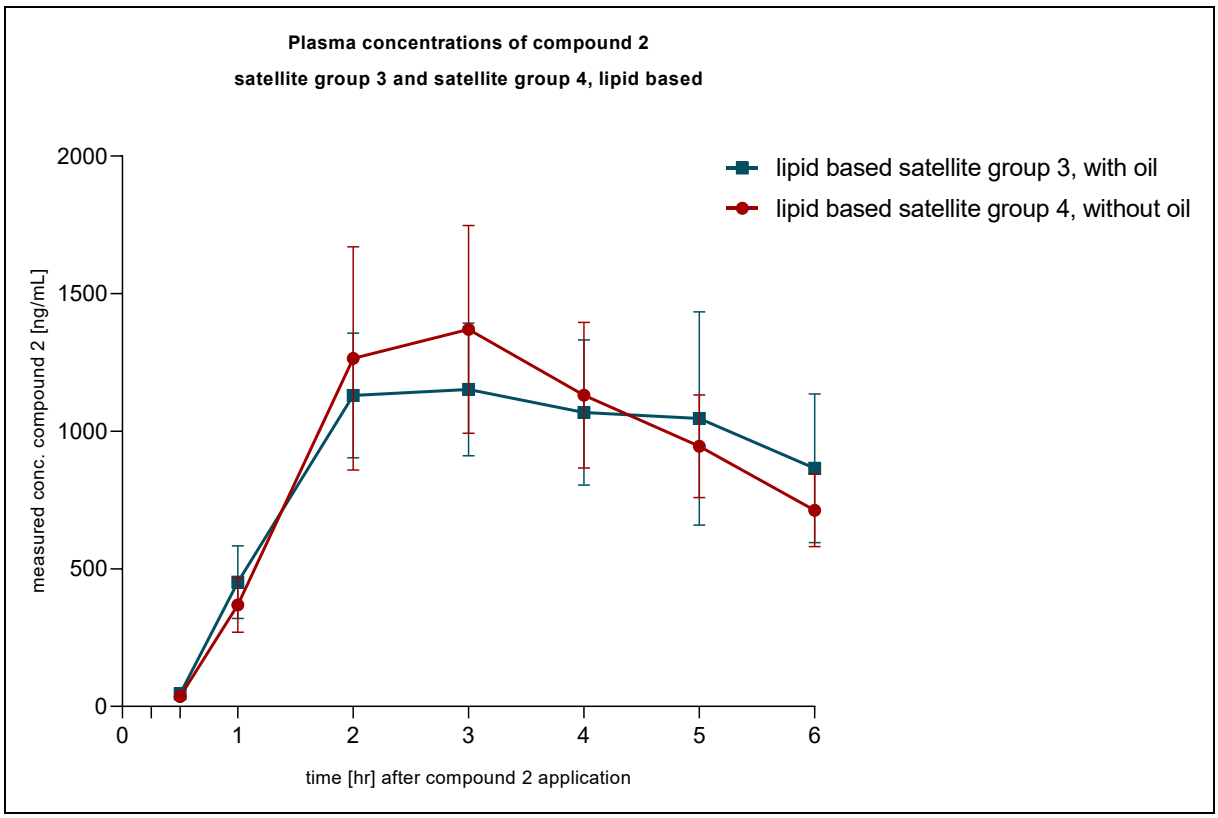


Figure 20: *Mean plasma concentration-time profiles of compound 2 in satellite animal groups lipid based formulation over 6 hours after compound administration.*

Comparison of the AUCs of satellite group 1 and satellite group 4 allows to assess the influence of olive oil pre-administration or the formulation in a lipid based vehicle on overall compound 2 exposure within 6 hours after administration. Due to lack of homogeneity the data were evaluated as log transformed and did not result in a significant difference (p = 0.2336) between the two groups (**Figure 21 and Table 15**).

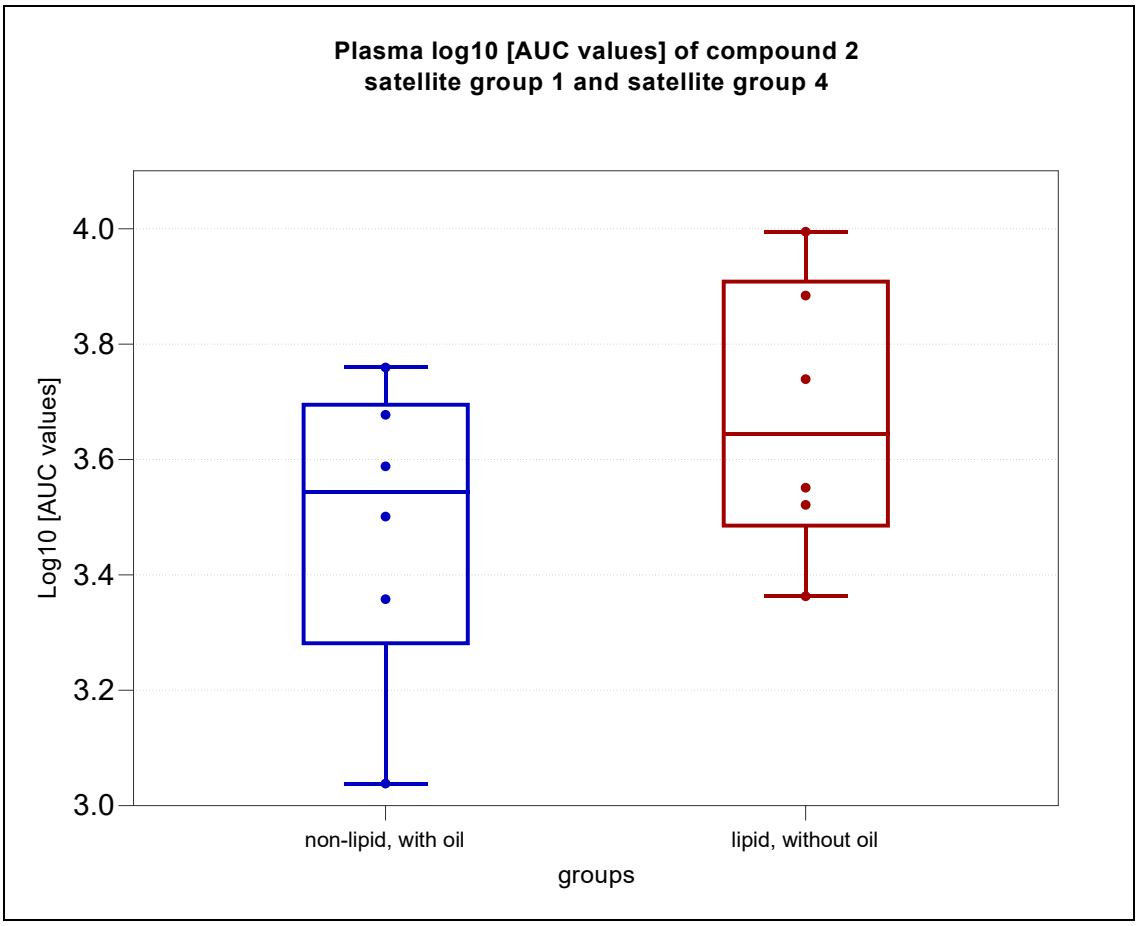


Figure 21: *The plasma AUC calculated for each animal individually of satellite animals group 1 and of satellite animals group 4, starting at the time point 1 hour after dose administration of compound 2.*

Fluid	Difference	Std Err Diff[a]	t Ratio	DF[b]	Prob > F
Plasma (non-lipid, with oil vs lipid, without oil)	1765.7	1370.6	1.288277	8.01424	0.2336

Table 15: *Statistical evaluation of the plasma AUC group 1 and plasma AUC group 4 mean difference after compound 2 administration. Comparison run using two-samples t-test.*

a. Std Err Diff: Standard error for the difference
b. DF: Degrees of freedom

Formulation influence

To assess the influence of the two formulations, the AUC in the lymph of groups 1 and 2 and the AUC in the plasma of the satellite animals of group 2 and of group 4 were compared (**Figure 22 and 23**). The data were log transformed due to inhomogeneity before analysis.

The AUC mean difference between the lipid based and non-lipid based formulations were statistically significant in the lymph of groups 1 and 2 (p = 0.0325) as well as in plasma of satellite group 2 and satellite group 4, p = 0.0297 (**Table 16**).

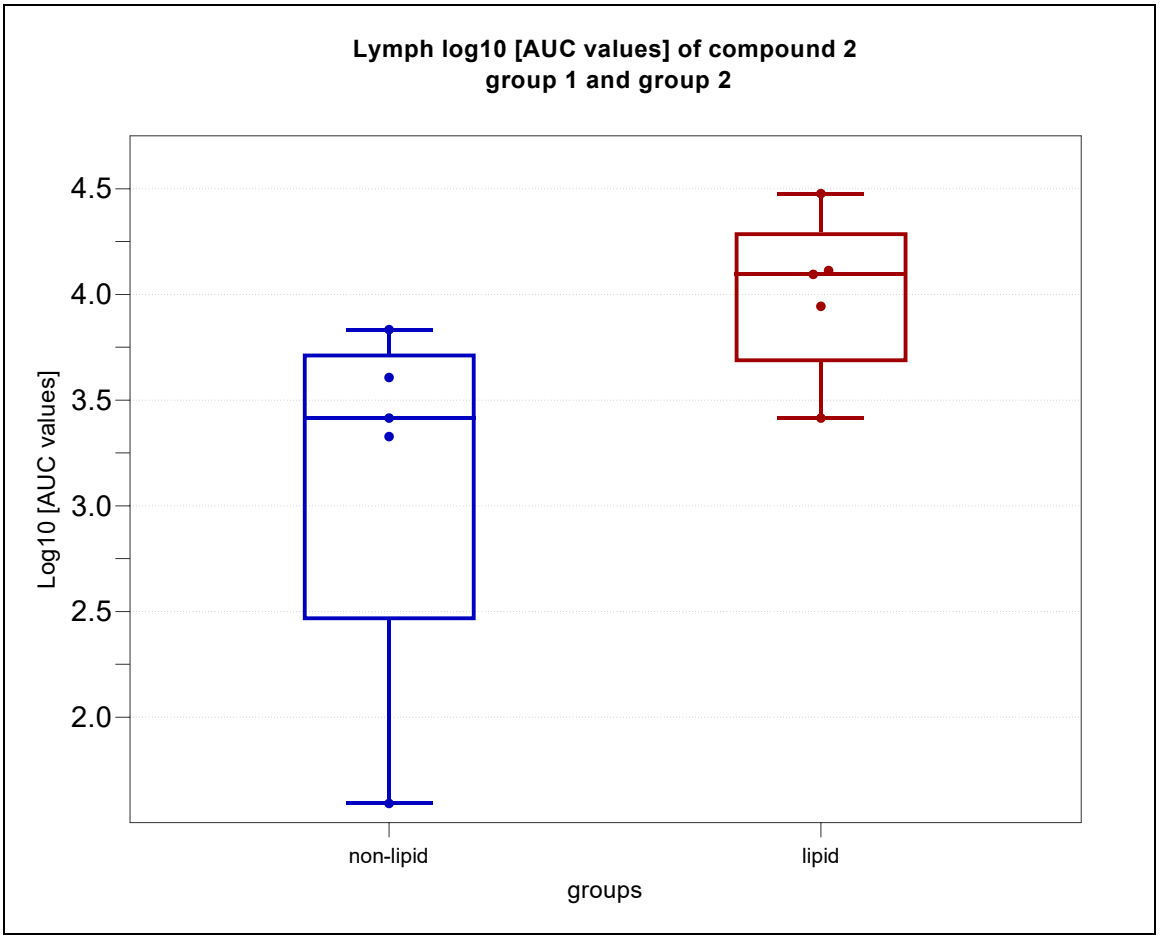


***Figure 22**: The lymph AUC calculated for each animal individually starting at the time point 1 hour after dose administration of compound 2.*

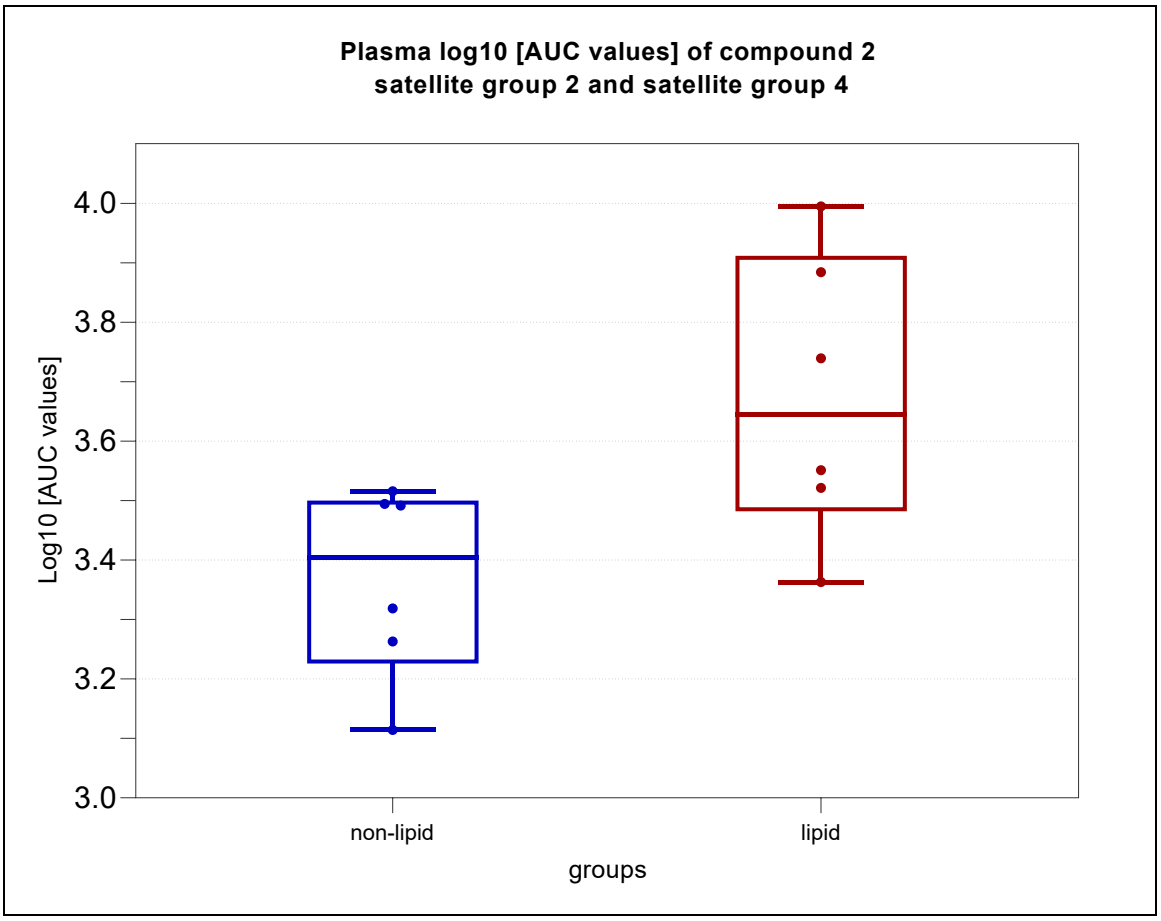


Figure 23: *The plasma AUC calculated for each animal individually starting at the time point 1 hour after dose administration of compound 2.*

Fluid	Difference	Std Err Diff[a]	t Ratio	DF[b]	Prob > F
Lymph (non-lipid vs lipid)	0.73335	0.31646	2.317381	18	0.0325*
Plasma (non-lipid vs lipid)	0.298952	10.118046	2.532514	10	0.0297*

Table 16: *Statistical evaluation of the lymph AUC groups, and plasma AUC groups mean difference after compound 2 administration. Comparison run using two-samples t-test (*represents $p \leq 0.05$).*

a. Std Err Diff: Standard error for the difference
b. DF: Degrees of freedom

2.2 Compound 3 – Itraconazole

Comparison of lymph kinetics in non-lipid and lipid based formulation (group 1 and group 2)

To measure concentration levels of compound 3 in the lymphatic fluid, a total number of 13 male rats underwent surgery with cannulation of the thoracic duct. After successful cannulation, 3 mg/mL of the substance was administered to six animals in a non-lipid based formulation, 10 % DMSO: 90 % PEG400, and further six animals in a lipid based formulation, 10 % PEG400: 10 % Cremophor EL: 80 % oleic acid. One animal was used as replacement rat. The lymph cannula tubing was placed in heparinized plastic vials for lymph collection for baseline, 15 minutes, 30 minutes and 1 hour collections over a 6-hour period. In three rats, continuous lymph flow could not be ensured over the entire period of 6 hours due to coagulated lymph in the catheter. The results of two other animals were not included in the evaluation as a consequence of incorrect administration. Due to the fasted sate of the animals, both walls of the stomach were accidently punctured and before noticing, a small amount formulation was administered intra peritoneally. **Figure 24** illustrates the mean lymph concentrations of both groups.

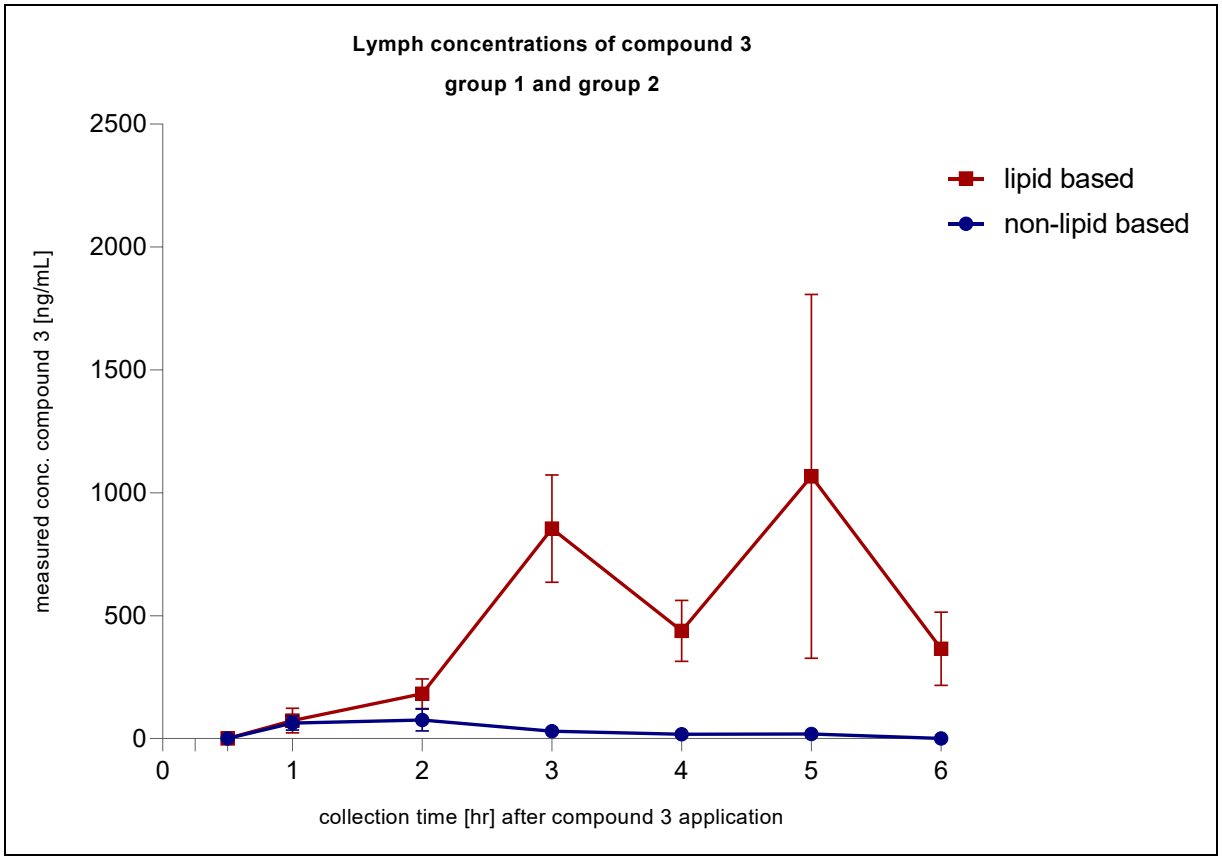


***Figure 24**: Mean concentrations of compound 3 in thoracic lymph fluid following oral administration with either a lipid based formulation (n = 4, red) or non-lipid based formulation (n = 4, blue) over 6 hours after compound administration.*

Compound 3 administered in the non-lipid based formulation showed only very low lymph concentrations. The C_{max} was already reached at 2 hours with a mean of 75.25 ng/mL. In contrast, the lipid based formulation led to an increase of the absorption rate of compound 3 into the lymph. A first peak was reached at 2-3 hours with 854 ng/mL, i.e. an approximately 11-fold higher concentraion compared to the non-lipid based formulation. After a decrease in concentration at 4 hours, a second peak could be observed at 5 hours with a mean measured lymph concentration of 1067.25 ng/mL.

Starting at collection time point 1 hour, the concentrations of compound 3 in the lymph of all animals from the lipid based group and the non-lipid group were compared at each single collection time point till 6 hours. The data were log transformed before analysis to fulfill the assumption of homogeneity of variance. While the concentrations of the two groups did not differ significantly during the first 2 hours the concentrations of the lipid based formulation group increased significantly from 3 hours onwards, with $p \leq 0.05$ (**Table 17**).

Time point	SS[a]	DFNum[b]	DFden[c]	F Ratio	Prob > F
1 hr	1.64	1	32	0.9721	0.3315
2 hrs	6.687	1	32	3.9422	0.0557
3 hrs	15.7	1	32	9.2562	0.0047*
4 hrs	15.64	1	32	9.2336	0.0049*
5 hrs	15.03	1	32	8.8611	0.0055*
6 hrs	27.21	1	32	16.0423	0.0003*

Table 17: *Statistical evaluation of lymph concentrations of compound 3, using repeated measures ANOVA, for time points 1 - 6 hours (*represents p ≤ 0.05).*

a. SS: Sum of squares
b. DFNum: Number of degrees of freedom
c. DFden: Degrees of freedom denominator

Influence of pre-administration of olive oil

Twelve animals received 0.1 mL of olive oil by oral gavage 1.5 – 2 hours before dosing. In order to assess whether the administration of olive oil has an influence on plasma concentrations and thus on oral bioavailability, the respective formulations of the satellite groups 1 and 2 were compared. **Figure 25** shows the comparison between the two non-lipid formulation groups, satellite group 1 and satellite group 2.

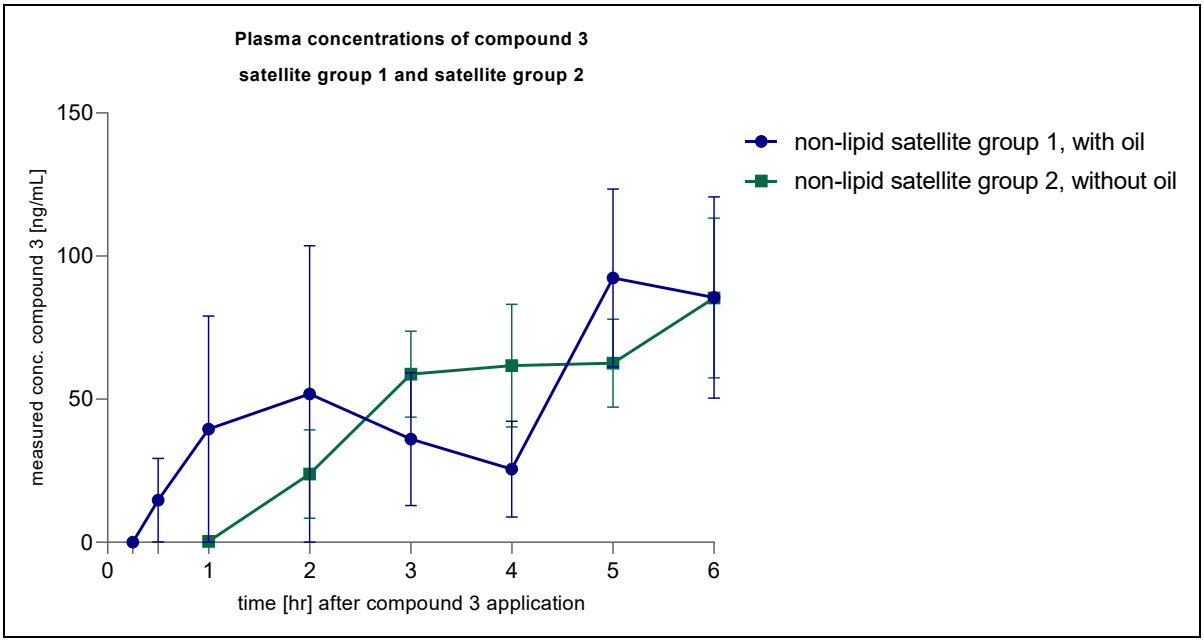


Figure 25: *The mean plasma concentration of satellite group 1 (blue) and 2 (green) over 6 hours after compound administration.*

The AUCs of the two groups show very similar values and accordingly no significant difference in the statistical analysis (**Figure 26 and Table 18**). Comparing the mean measured concentration of both groups in plasma, a slight increase in the first concentrations of the satellite animal group 1 is notable within the first 2 hours, starting already 15 minutes after compound administration. Also concentrations of satellite group 2, only receiving the non-lipid formulation, show an increase, starting from the first hour. There was no statistically significant difference between the mean plasma concentrations of the two groups during the entire time course.

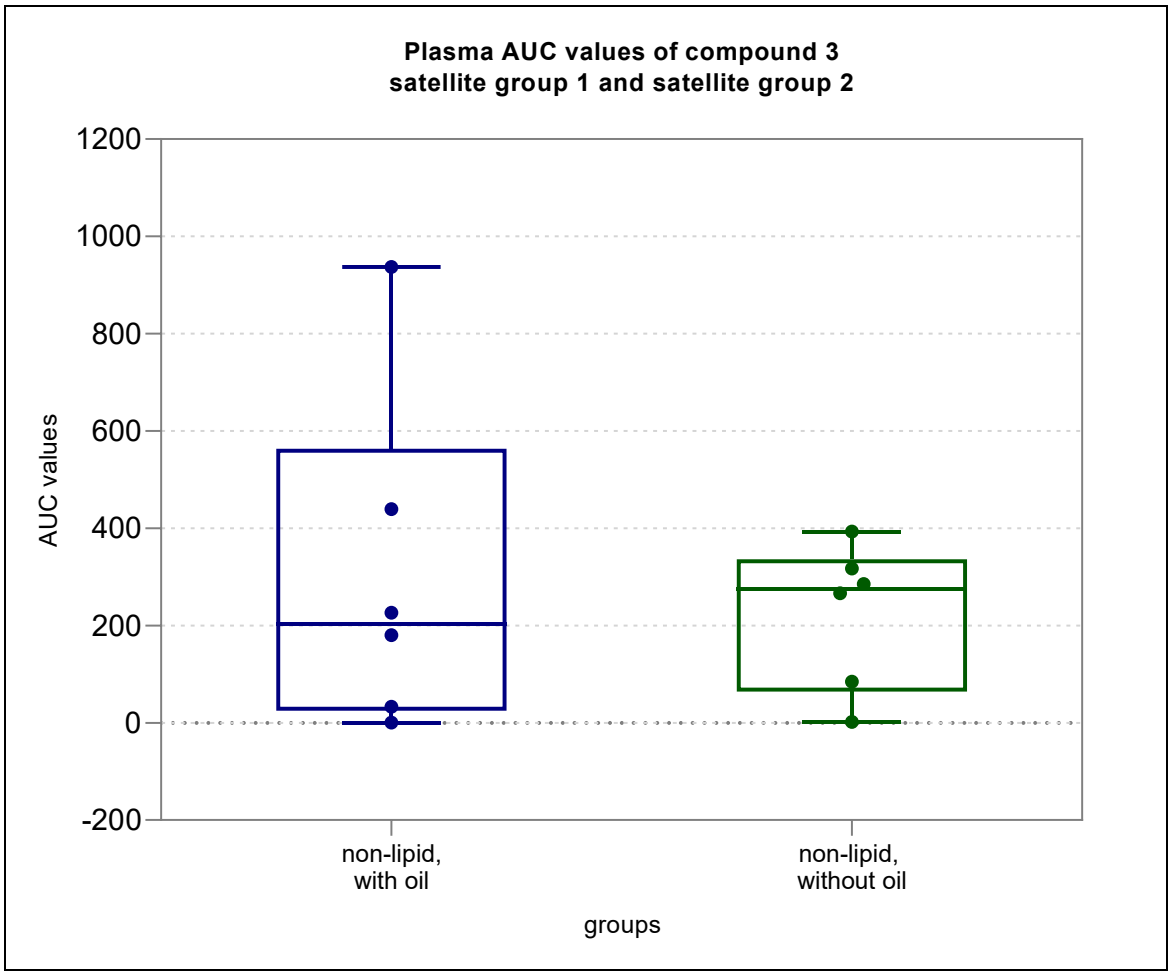


***Figure 26**: The AUC in plasma calculated for each animal individually starting at the time point 1 hour after dose administration of satellite group 1 (blue) and 2 (green).*

Formulation	SS[a]	DFNum[b]	DFden[c]	F Ratio	Prob > F
Plasma (non-lipid with oil vs non-lipid without oil)	1088	1	20	0.0157	0.9016

***Table 18:** Statistical evaluation of the plasma AUC non-lipid groups mean difference. Comparison run using two-way ANOVA.*

a. SS: Sum of squares
b. DFNum: Number of degrees of freedom
c. DFden: Degrees of freedom denominator

Looking at the individual animal curves of satellite groups 1, there is evidence that the progression of the mean measured concentration curve during the first 3 hours can be traced back to a single animal. The first peak and the high variance can be explained by the concentrations measured in plasma of the remaining five animals, which are below the LLOQ of 26.9 ng/mL. Excluding animal number 25, illustrated in **Figure 27**, shifts the development of the curve with only one peak at 5 hours. Even though all animals were fasted for the same duration, of the 6 animals, only this one outlier was found to have a completely empty

stomach. However, the outlier was considered in the statistical evaluation and only excluded for the presentation of the overall curve in order to see its effect on the results.

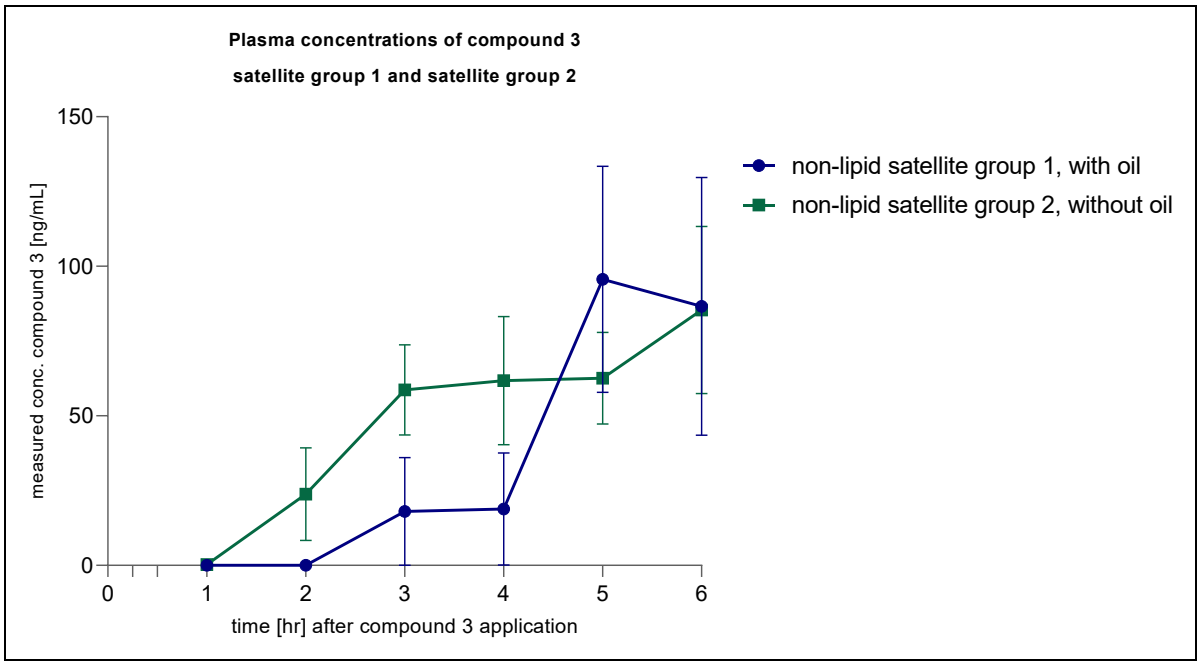


Figure 27: *Mean concentrations in plasma of satellite group 1 (blue) and 2 (green), excluding animal 25 over 6 hours after compound administration.*

Comparing the plasma concentration curves of the lipid based formulation groups, a similar curve shape is observed (**Figure 28**). Both groups show a steady increase in mean plasma concentrations over the entire 6-hour period. In satellite animals group 2, administered with the formulation only, this increase is steeper and only moderated between the 4- and 5-hour collection time points. On the other hand, the curve of satellite group 1, which received olive oil before the substance was administered, runs exponentially and starts to increase more precipitously at 3 hours. However, the mean plasma concentration values are higher in the satellite group that received only the formulation, compared to the group that was administered with olive oil first. This increase in concentrations also becomes evident when evaluating the AUCs. The statistical analysis resulted in a significant difference between these two groups (**Table 19 and Figure 29**).

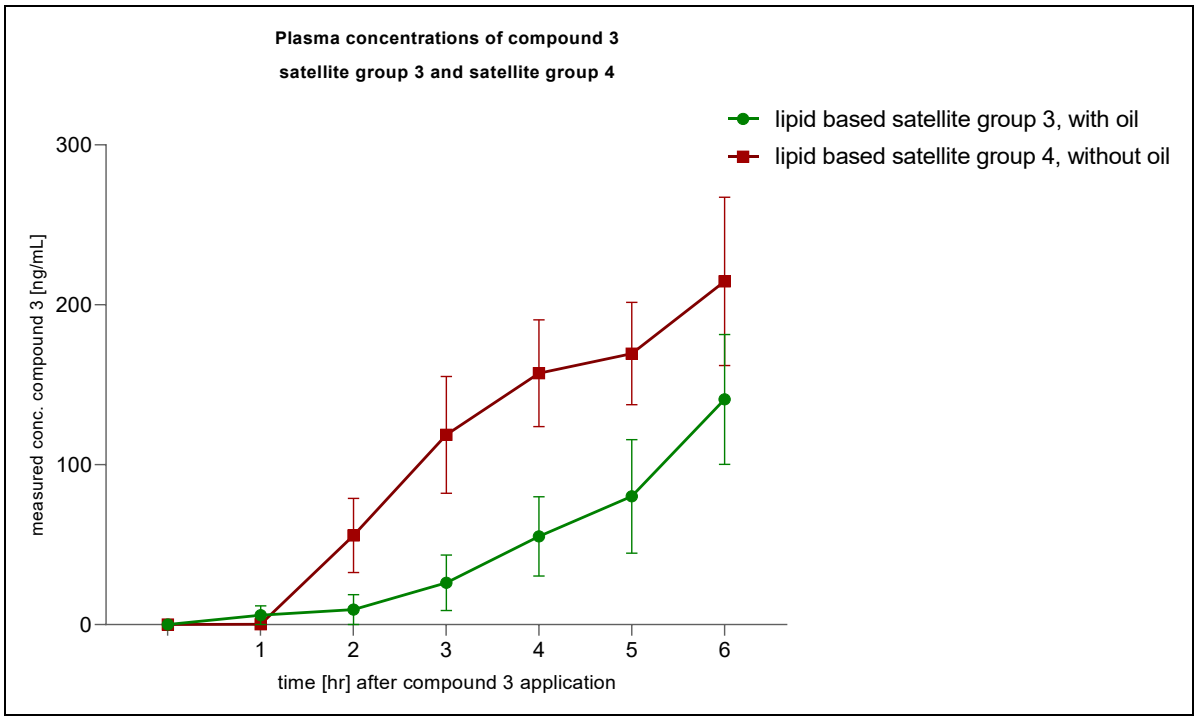


***Figure 28**: The mean concentrations in plasma of satellite group 3 (green) and 4 (red) over 6 hours after administration.*

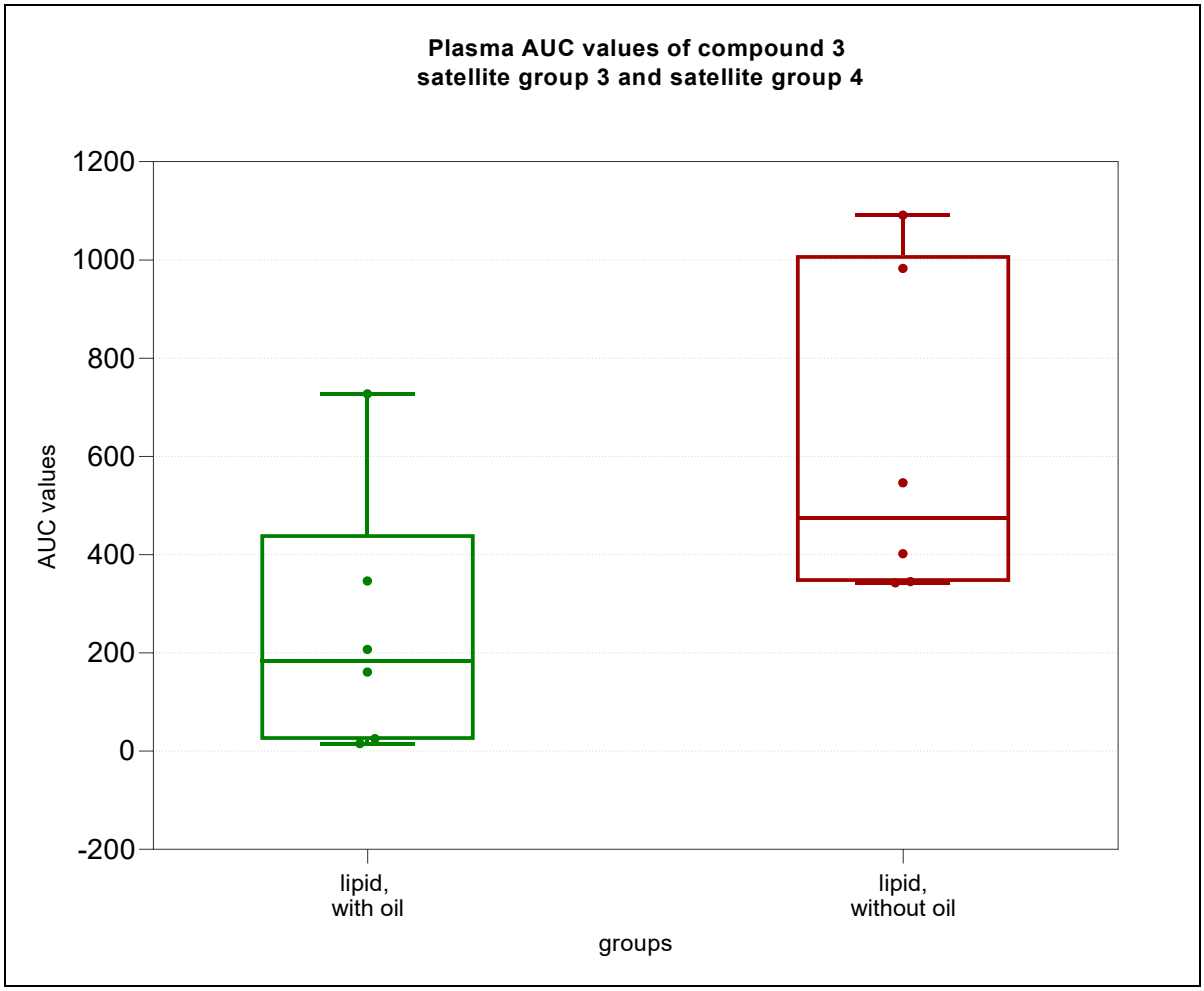


***Figure 29**: The AUC in plasma calculated for each animal individually starting at the time point 1 hour after dose administration of satellite group 3 (green) and 4 (red).*

Formulation	**SS[a]**	**DFNum[b]**	**DFden[c]**	**F Ratio**	**Prob > F**
Plasma (lipid based with oil vs lipid based without oil)	4e+5	1	20	5.7286	0.0266*

Table 19: *Statistical evaluation of the plasma AUC lipid based groups mean difference. Comparison run using two-way ANOVA (*represents p ≤ 0.05).*

a. SS: Sum of squares
b. DFNum: Number of degrees of freedom
c. DFden: Degrees of freedom denominator

Comparison of plasma kinetics in non-lipid and lipid based formulation (satellite group 2 and satellite group 4)

In order to show the formulation effect in plasma, 12 animals were treated with 3 mg/kg of compound 3 orally either in the lipid based or non-lipid based formulation, without undergoing surgery. The plasma concentrations of compound 3 were measured in blood samples, collected at baseline, 15, 30 minutes and 1, 2, 3, 4, 5 and 6 hours. Mean plasma concentrations of these groups, satellite animal group 2 and satellite animal group 4, are compared in **Figure 30**. In both groups measurable first values were obtained at 1 hour. Starting at 2 hours, higher concentrations could be measured in the lipid based group over the entire course of the experiment. Both groups did not reach a peak during the 6 hours but showed a more pronounced increase in the time between hours 5 and 6. The maximum mean measured concentration of compound 3 in the non-lipid based formulation group was 85.38 ng/mL, while the maximum mean concentration of the lipid based formulation group was 214.7 ng/mL, both at 6 hours. The difference between the concentrations of the lipid based and non-lipid based group was significant at 5 and 6 hour time points with p ≤ 0.05 (**Table 20**).

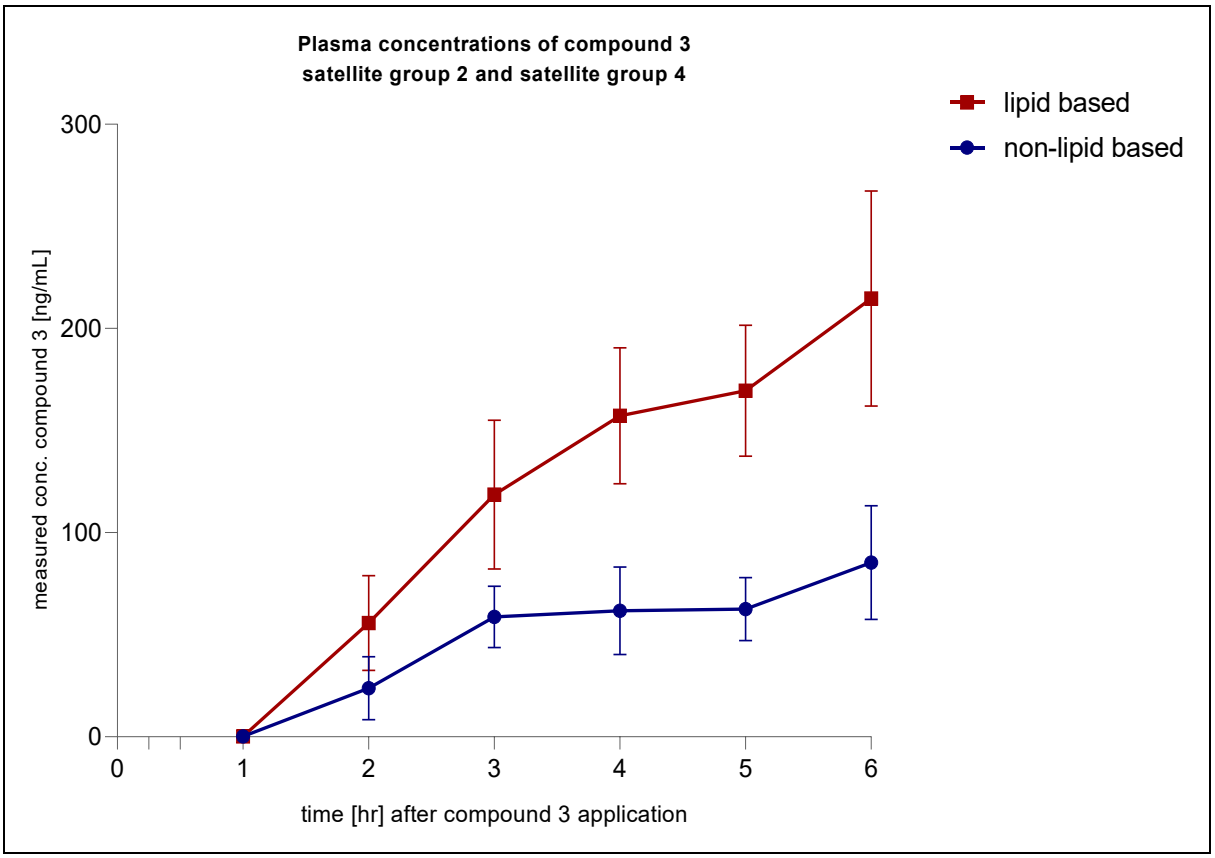


***Figure 30**: Mean plasma concentration-time profiles of compound 3 following oral administration either in a lipid based formulation (n = 6, red) or non-lipid based formulation (n = 6, blue) over 6 hours after administration.*

Time point	SS[a]	DFNum[b]	DFden[c]	F Ratio	Prob > F
1 hr	6e-28	1	52	0.0000	1.0000
2 hrs	975.6	1	52	0.2195	0.6414
3 hrs	6354	1	52	1.4295	0.2373
4 hrs	10036	1	52	2.2581	0.1390
5 hrs	24355	1	52	5.4797	0.0231*
6 hrs	34374	1	52	7.7339	0.0075*

***Table 20:** Statistical evaluation of concentrations of compound 3 in plasma, using repeated measures ANOVA, for time points 1 - 6 hours (*represents $p \leq 0.05$).*

a. SS: Sum of squares
b. DFNum: Number of degrees of freedom
c. DFden: Degrees of freedom denominator

To assess the influence of the two formulations over the entire time course of 6 hours, the AUC in the lymph of groups 1 and 2 and the AUC in the plasma of the satellite animals of groups 2 and 4 were also determined (**Figure 31 and 32**). The data were evaluated as log transformed to fulfill the assumption of homogeneity.

The AUC mean difference between the lipid based and non-lipid based formulations were statistically significant in the lymph of groups 1 and 2 ($p = 0.0179$) as well as in plasma of satellite group 2 and satellite group 4, $p = 0.02046$ (**Table 21**).

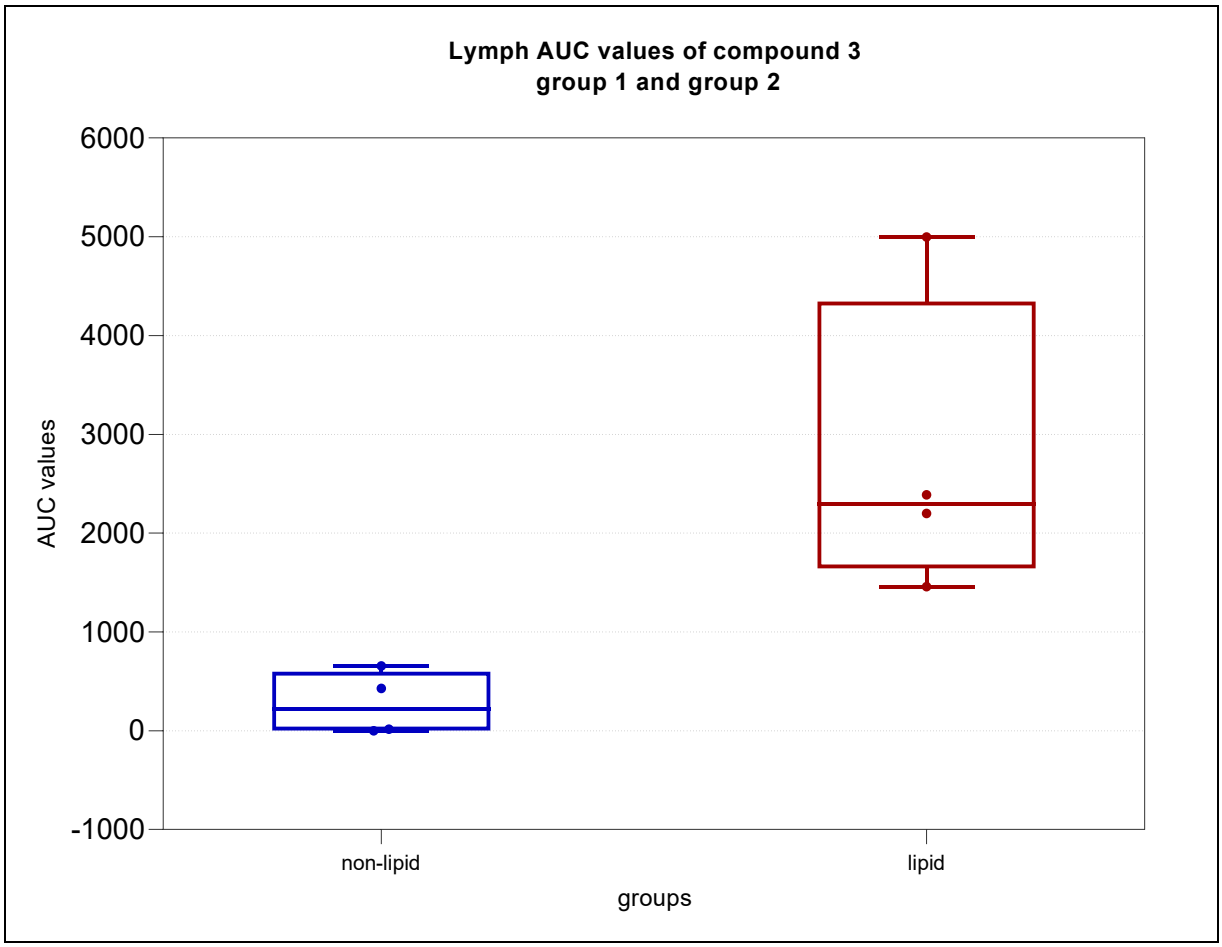


Figure 31: *The lymph AUC calculated for each animal individually starting at the time point 1 hour after dose administration of compound 3 of group 1 (blue) and group 2 (red).*

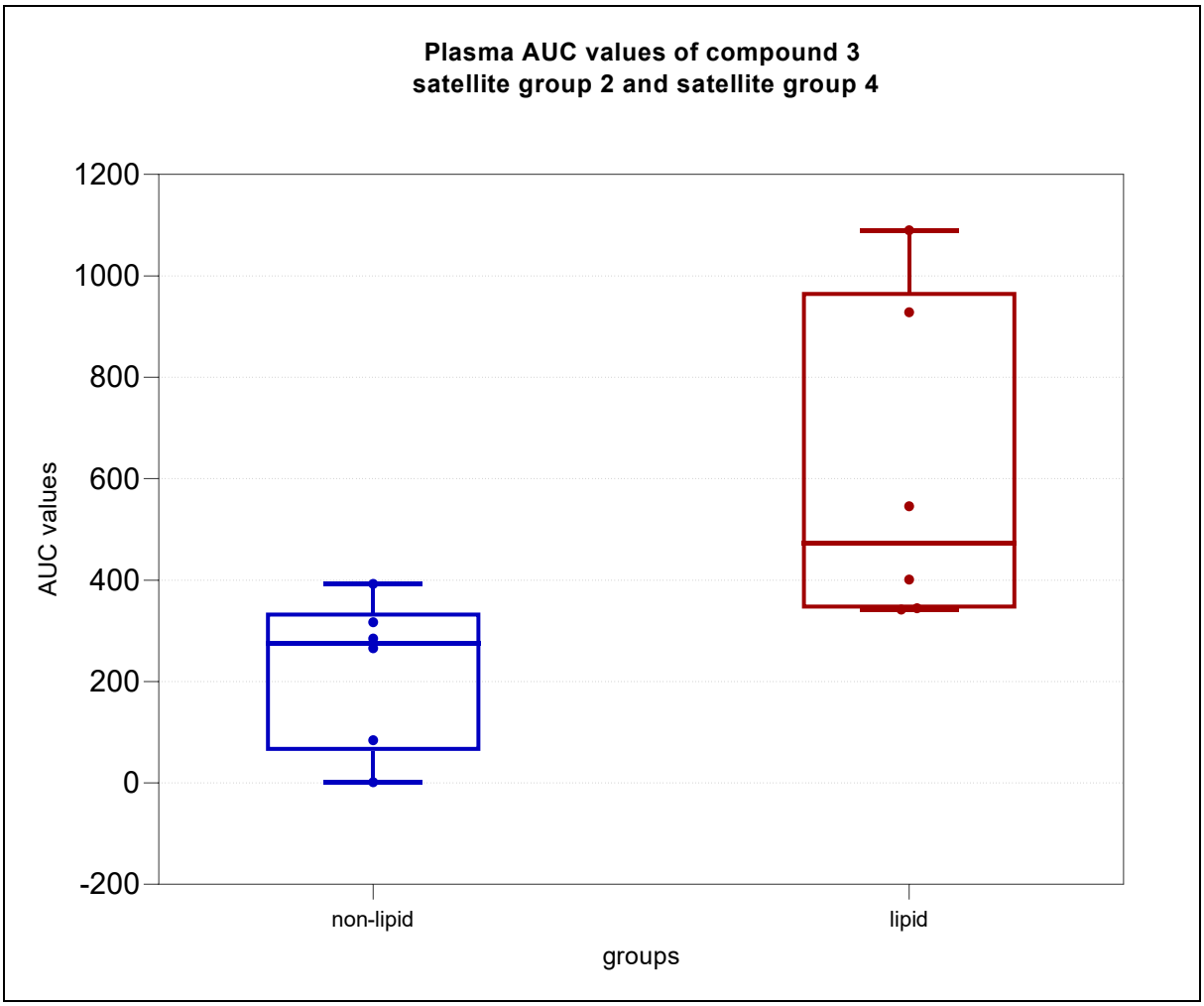


***Figure 32**: The plasma AUC calculated for each animal individually starting at the time point 1 hour after dose administration of compound 3.*

Fluid	SS[a]	DFNum[b]	DFden[c]	F Ratio	Prob > F
Lymph (non-lipid vs lipid)	8.495	1	16	6.9546	0.0179*
Plasma (non-lipid vs lipid)		1	7.6548	5.7561	0.0046*

***Table 21**: Statistical evaluation of the lymph AUC groups, and plasma AUC groups mean difference after compound 3 administration. Comparison run using two-way ANOVA (*represents $p \leq 0.05$).*

a. SS: Sum of squares
b. DFNum: Number of degrees of freedom
c. DFden: Degrees of freedom denominator

3 Immunology experiments

3.1 Lymph smear

A lymph smear was prepared to get an overview of the cell composition of the lymph. Histological evaluation showed several structurally similar cells in the wright-stained lymph smear with a prominent cell nucleus and with a scanty cytoplasm representing lymphocytes. In addition, there are many fat droplets in various sizes around the cells (**Figure 33**).

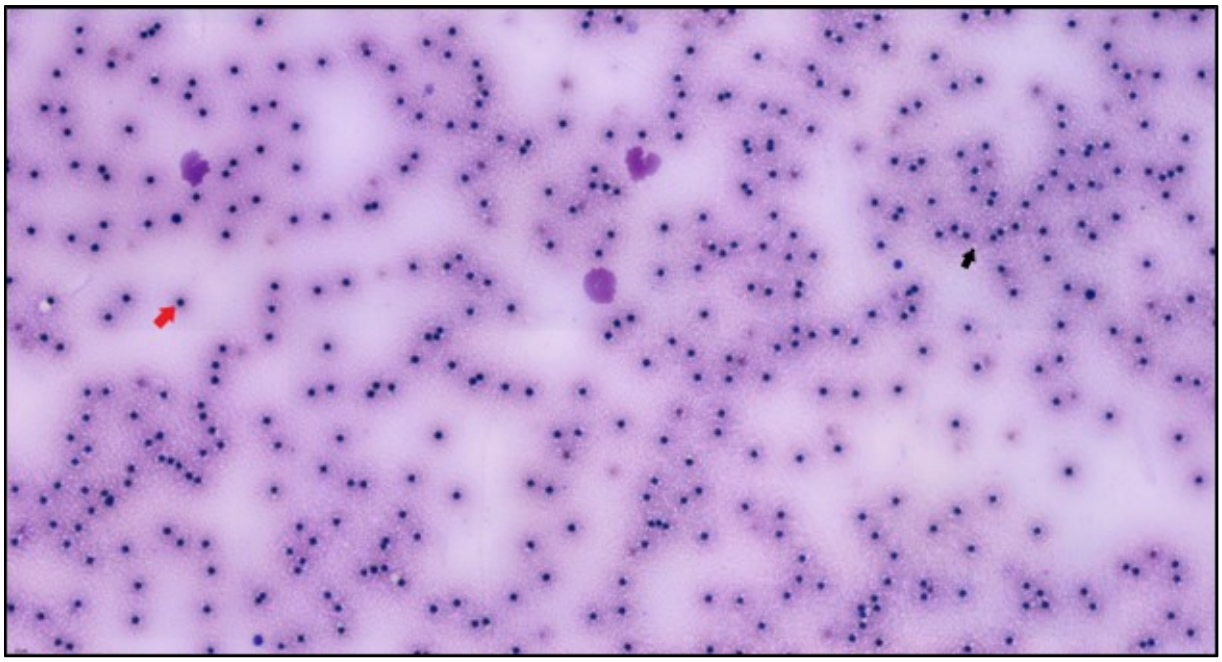


Figure 33: *Wright-stained lymph smear (20-fold magnification), with lymphocytes (red arrow) and fat droplets (black arrow).*

3.2 *In-vitro* pilot study

The *in-vitro* study was carried out to determine whether the antibody chosen, the selected staining pattern and the FACS panel settings are suitable for the *in-vivo* study. In order to ensure that cells are not dying during the one-hour incubation period, the experiment was carried out in centrifuged samples with DMEM high-glucose medium in addition to untreated EDTA blood samples. The death cell staining CD8a of FACS analysis showed more than 99.70 % live cells in all samples, indicating very low ratio of cell death (**Figure 34**).

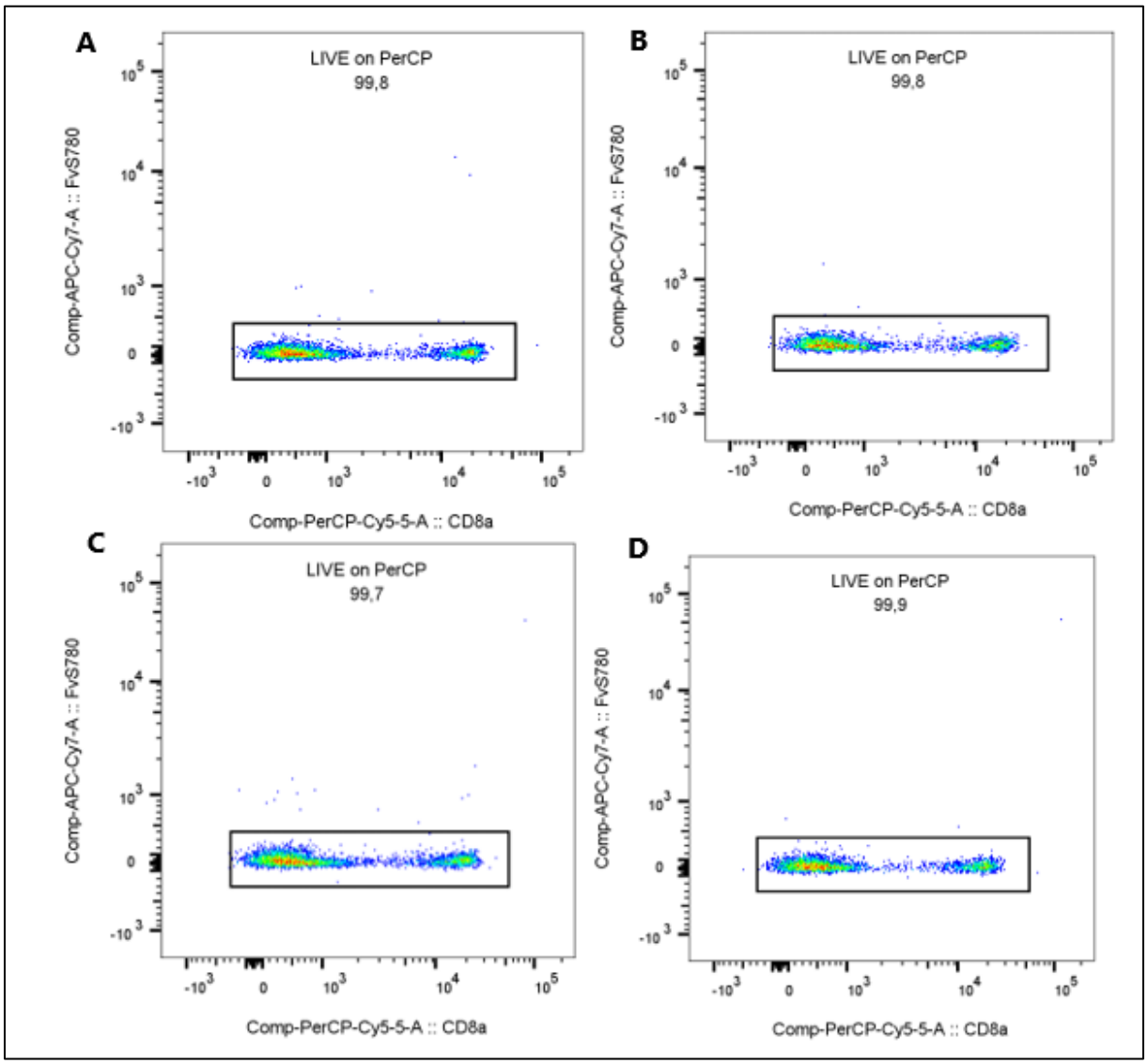


Figure 34: *Live cells gating in-vitro study of (A) untreated blood sample, (B) blood sample with mAb, (C) blood sample with DMEM high glucose medium and (D) blood sample with DMEM high glucose medium and mAb.*

After adding the antibody and subsequent incubation for one hour, a decrease in the number of T-cells and lymphocytes was seen compared to the samples without antibody. The T-cell population decreased on average by 48.55 % and the lymphocytes by 13.60 % (**Figure 35 and Table 22**).

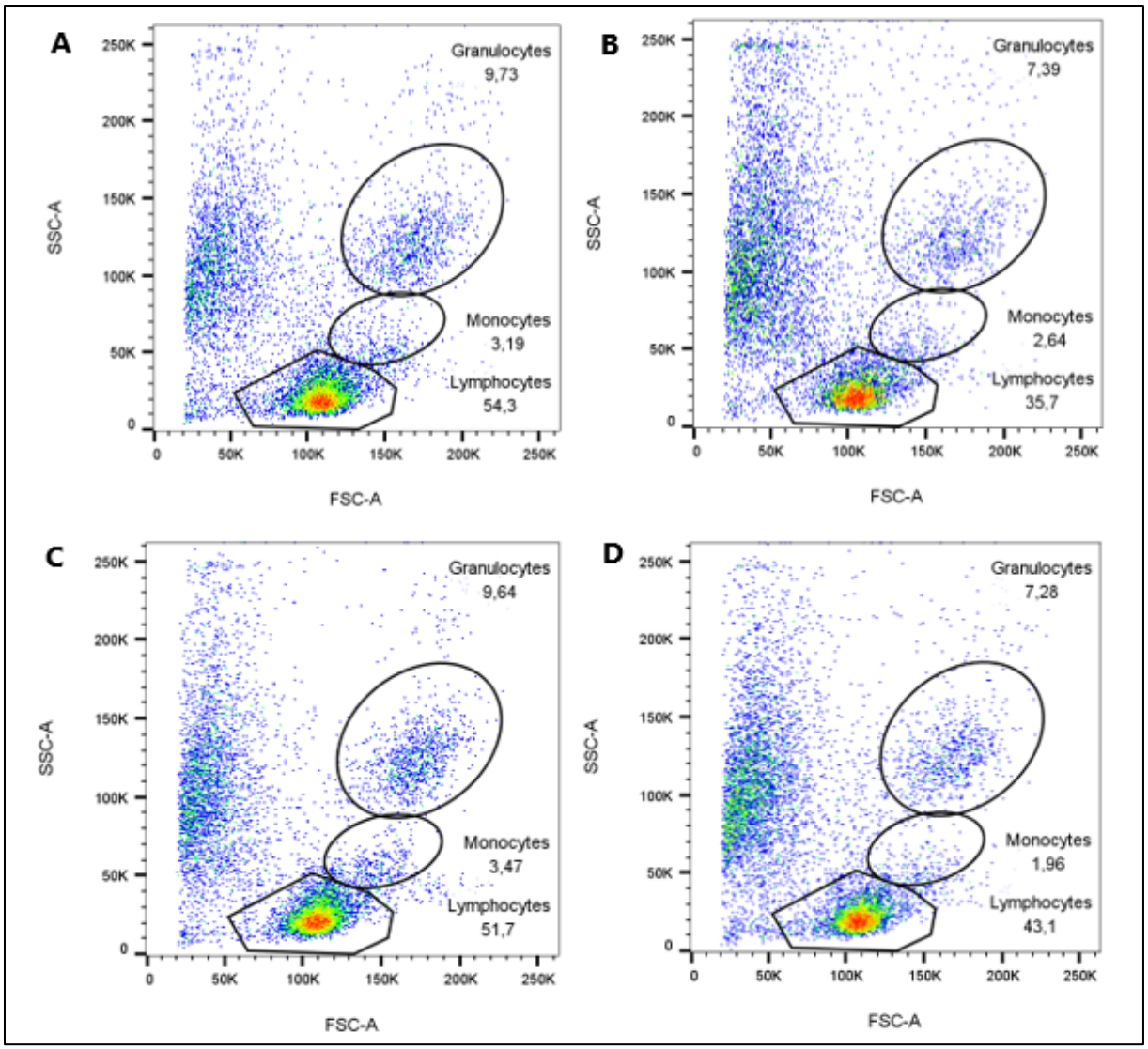


Figure 35: *Cell gating in-vitro-pilot study of (A) untreated blood sample, (B) blood sample with mAb, (C) blood sample with DMEM high glucose medium and (D) blood sample with DMEM high glucose medium and mAb.*

	T-cells [%]	**Lymphocytes [%]**
EDTA sample	41.60	54.30
EDTA sample + mAb R73	1.78	35.70
EDTA sample + DMEM high glucose medium	58.90	51.70
EDTA sample + DMEM high glucose medium + mAb R73	1.63	43.10

Table 22: *Lymphocytes and T-cells measured in the samples by FACS analysis.*

3.3 Compound 4 - R73 monoclonal antibody (mAb)

Raw data from FACS analysis (BD FACSVerse™ Cell Analyzer, BD Biosciences) were summarized in an Excel Sheet, including the creation of dot plots, by the Analytical Platform, PCS unit, AbbVie Deutschland GmbH Co. & KG, Ludwigshafen. These data were submitted to the Data and Statistical Science (DSS) department at AbbVie Deutschland GmbH Co. & KG, Ludwigshafen. Cell compositions of all time points were considered appropriate for all subsequent statistical analysis.

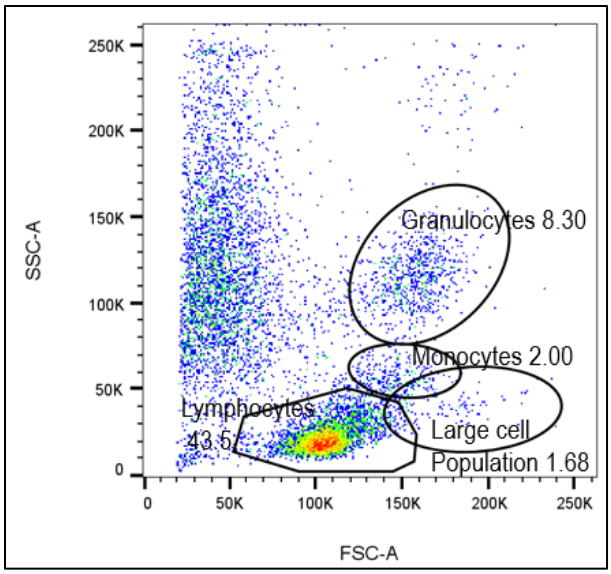
The *in-vivo* pilot study to assess potential immunological changes after subcutaneous antibody administration was conducted with compound 4 in 12 male rats. Six animals were assigned to group 1 and were used exclusively for the collection of blood samples at baseline, 15, 30 minutes and 1, 2, 3, 4 and 24 hours after compound administration. The other six animals, (group 2) were surgically prepared for collection of lymphatic fluid. After collection of baseline lymph, further time points of the collection time frame were 15, 30 minutes and 1, 2, and 3 hours after compound administration. Additionally, baseline and terminal blood was collected from animals of group 2. Lymph samples from five animals could be completely analyzed and evaluated. One animal was excluded because of interrupted lymph flow. Additionally, baseline and terminal blood values were collected from operated animals.

Comparison of cell populations in baseline lymph and baseline blood of group 2 (lymph cannulated animals)

Cell populations that can be measured in blood and lymph samples with the BD FACSVerse™ Cell Analyzer, BD are shown in **Figure 36 and 37** according to their morphology in the stream. Significantly more lymphocytes ($p < 0.0001$) could be measured in the lymph than in blood with average amounts of 96.28 % and 47.70 % of total blood cells, respectively (**Figure 38**). In comparison, the mean proportion of granulocytes (0.05 %) and monocytes (0.17 %) in the lymph was significantly ($p = 0.0019$ and $p = 0.0003$) lower than in the blood, where 9.32 % granulocytes and 2.82 % monocytes were detected (**Figure 39 and 40**). In addition, it was noticed that no erythrocytes could be measured in any of the lymph samples.

Cell size

When comparing the relative cell size of lymphocytes in lymph with lymphocytes in blood using the flow cytometer [median forward scatter A (FSC-A)], it is noticeable that the lymphocytes in the lymph are significantly ($p = 0.0185$) smaller, by 16.00 % on average. This can be seen by comparing the two dot plots (**Figure 36 and 37**). The center of the plot of cells in blood is shifted further to the right on the x-axis than the colored center of the cloud of cells in lymph.



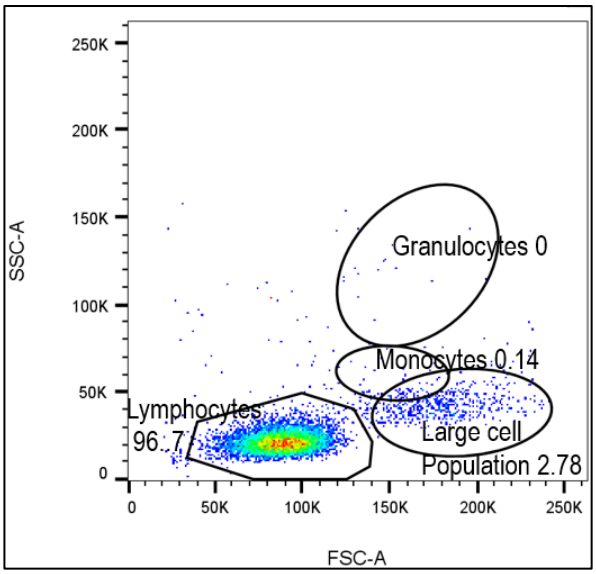


Figure 36 *(left)**:** Dot plot, forward scatter A (FSC-A) vs side scatter A (SSC-A) showing the distribution of cells based upon size in one exemplary baseline blood sample of animal 1 of group 2. The different white blood cell populations are indicated by circles. Erythrocytes can be seen at the left side of the X axis, along the Y-axis.* ***Figure 37*** *(right)**:** Baseline lymph sample exemplary of animal 1 group 2.*

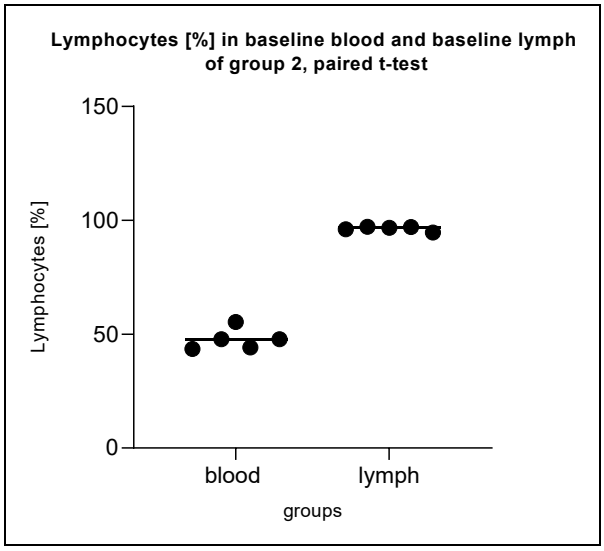


Figure 38: *Significantly ($p < 0.0001$) higher amount of lymphocytes in baseline lymph samples compared to baseline blood samples in group 2 ($n = 5$). A paired t-test was used to calculate the significance level.*

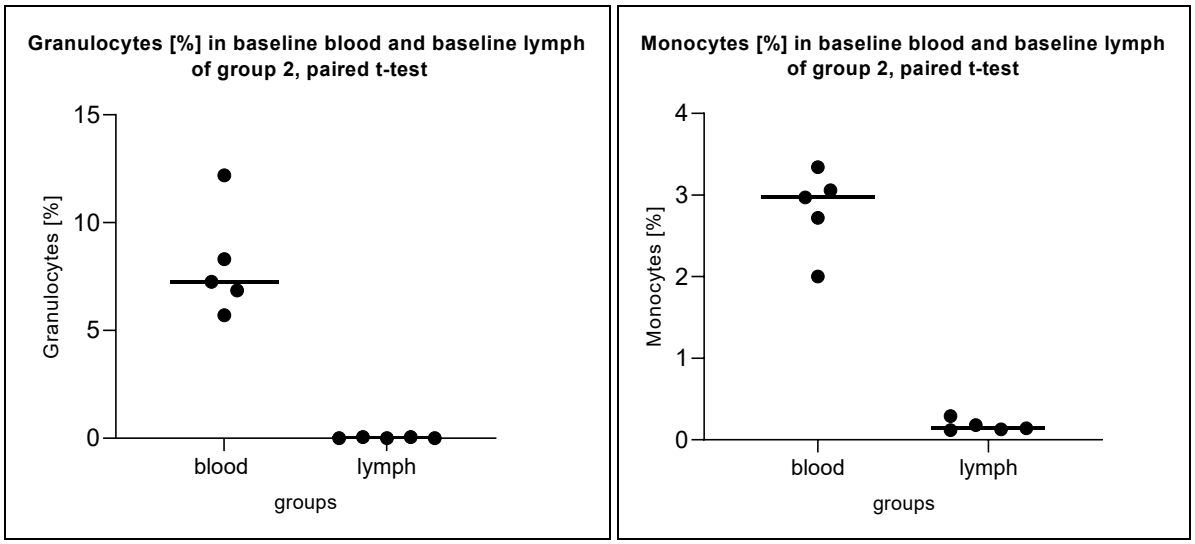


Figure 39 *(left)**: Significantly (p = 0.0019) higher amount of granulocytes and* ***Figure 40*** *(right)**: Significantly (p = 0.0003) higher number of monocytes in baseline blood samples compared to baseline lymph samples of group 2 (n = 5). A paired t-test was used in both cases to calculate significance levels.*

In addition, subpopulations of lymphocytes, such as T-cells, B-cells, NK-cells, NK T-cells, T-helper cells and T-killer cells were identified in both, blood and lymph (**Table 23**).

Cell population or subpopulation	**Mean baseline blood (group 2)**	**Mean baseline lymph (group 2)**
Lymphocytes [%]	47.70	96.28
Granulocytes [%]	9.32	0.05
Monocytes [%]	2.82	0.17
T-cells [%]	50.26	90.02
B-cells [%]	37.96	8.19
NK-cells [%]	3.18	0.10
NK T-cells [%]	2.23	4.49
T-killer cells [%]	28.22	22.10
T-helper cells [%]	69.32	76.04

Table 23: *Cell populations and subpopulation analyzed with the BD FACSVerse™ Cell Analyzer, BD Biosciences in baseline values of blood and lymph in animals of group 2.*

While significantly more T-cells and T-helper cells could be measured in the lymph (**Figure 41 and 42**), more B-cells, NK-cells and T-killer cells could be analyzed in the blood (**Figure 43, 44 and 45**). In the case of the NK T-cells, there was no significant difference between blood and lymph (**Figure 46**).

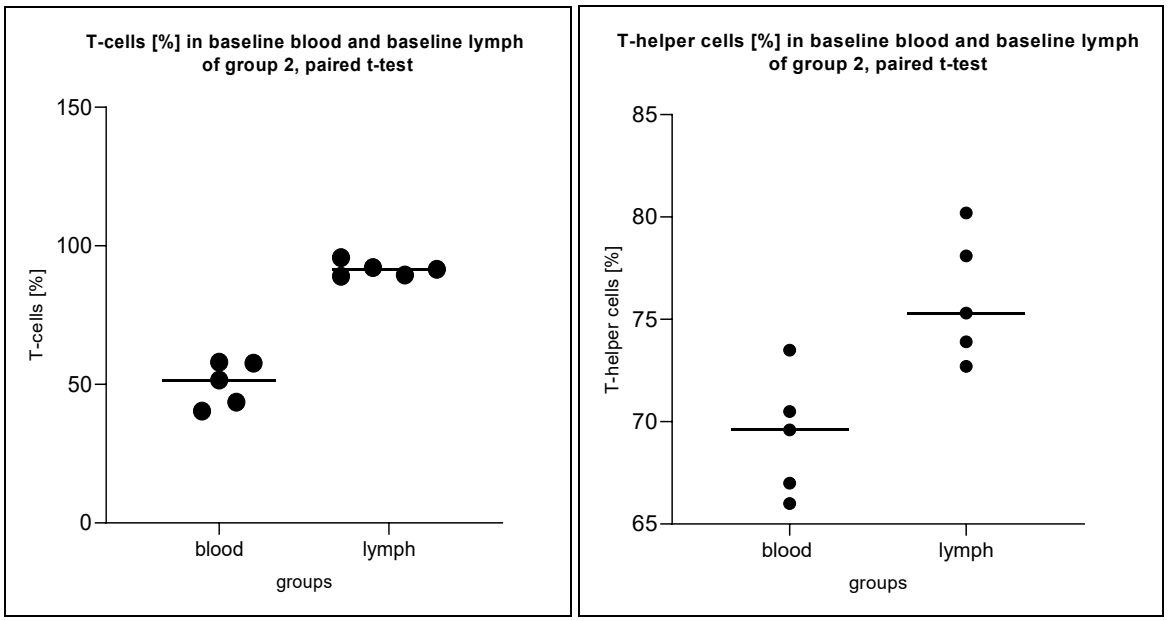


***Figure 41** (left): Significantly (p = 0.0002) higher amount of T-cells and **Figure 42** (right): Significantly (p = 0.0322) higher amount of T-helper cells in baseline lymph samples compared to baseline blood samples in group 2 (n = 5). A paired t-test was used in both cases to calculate significance levels.*

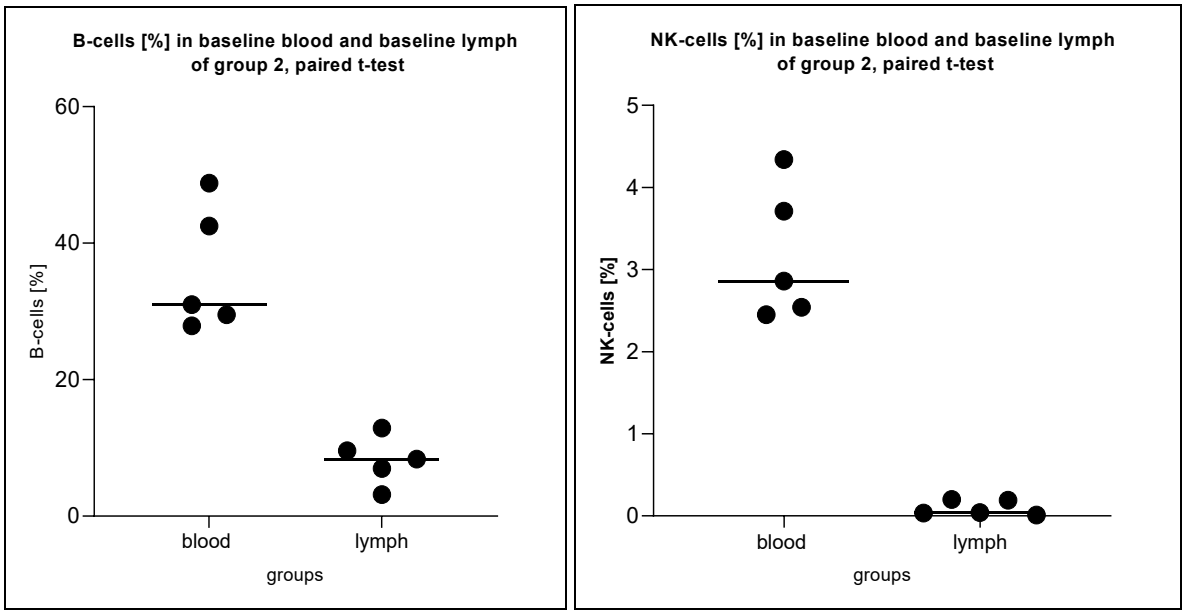


***Figure 43** (left): Significantly (p = 0.0007) higher amount of B-cells and **Figure 44** (right): Significantly (p = 0.0012) higher amount of NK-cells in baseline blood compared to baseline lymph in group 2 (n = 5). A paired t-test was used in both cases to calculate significance levels.*

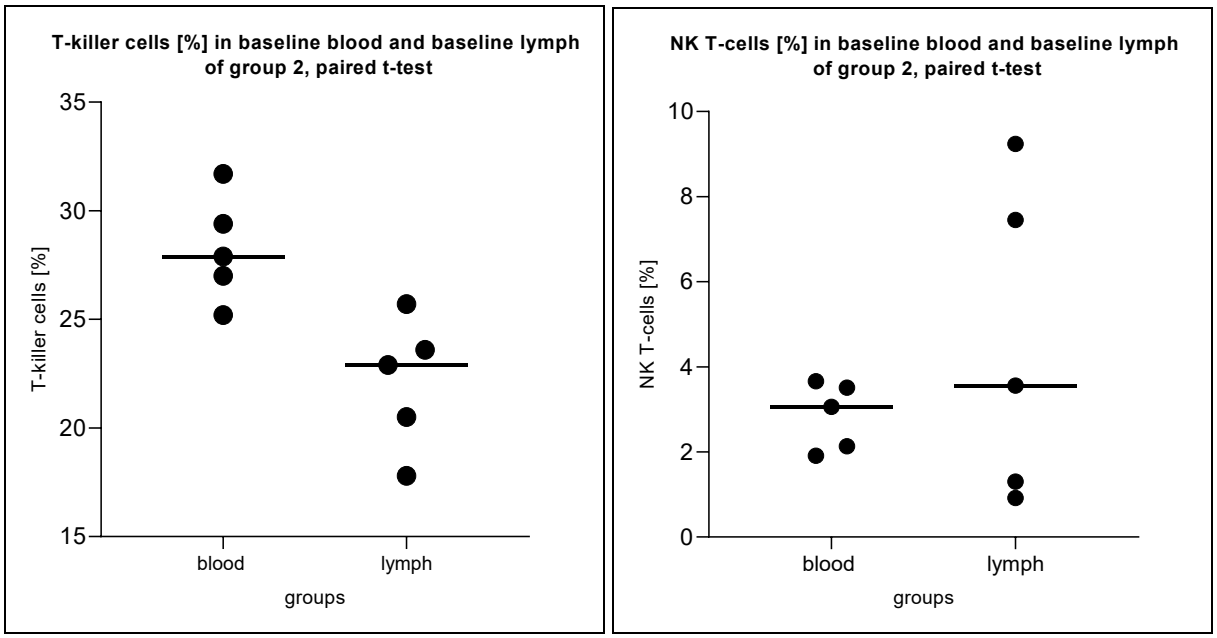


***Figure 45** (left): Significantly(p = 0.0279) higher amount of T-killer cells in baseline blood compared to baseline lymph in group 2 (n = 5) using a paired t-test and **Figure 46** (right): No significant (p = 0.345) difference between NK T-cells in baseline blood samples and baseline lymph samples of group 2 (n = 5) using paired t-test.*

Influence of compound 4 on the cell populations in blood and lymph (group 1 vs group 2)

Due to unexplained cell degradation in samples from animal 10 and animal 8 of group 1, the values of these two animals were not included in the statistical analysis. The live / death cell staining CD8a in the FACS analysis showed an average of 99.65 % live cells in all used samples, indicating a very low proportion of dead cells. Therefore, samples were considered as suitable for further analysis.

To evaluate the influence of compound 4 on white blood cell (sub)populations, the difference in percentage between the baseline and the 3 hours post dose values were calculated and compared to the group 2 difference between baseline and 3 hours post dose.

This comparison showed that the amount of T-cells decreased significantly following treatment with compound 4 in non-lymph cannulated animals. In blood samples of animals without surgery, group 1, a significant (p = 0.0236) T-cell decrease can be measured over 3 hours, while in the blood samples of lymph cannulated animals, group 2, the difference between the baseline and the 3-hour value was non-significant (p = 0.2231). Using the unpaired t-test, a significant (p = 0.0018) difference between group 1 and group 2 in changes of the T-cell population in baseline and 3-hour blood samples could be measured (**Figure 47**).

A similar difference can be observed in the NK T-cell population. While NK T-cells are significantly (p = 0.0354) decreased in samples collected 3 hours post dose compared to

baseline samples of group 1 animals, no statistically significant (p = 0.345) change is observed between blood samples of group 2 animals. When comparing the difference values of group 1 and group 2, a statistic significance (p = 0.0136) could be determined (**Figure 48**).

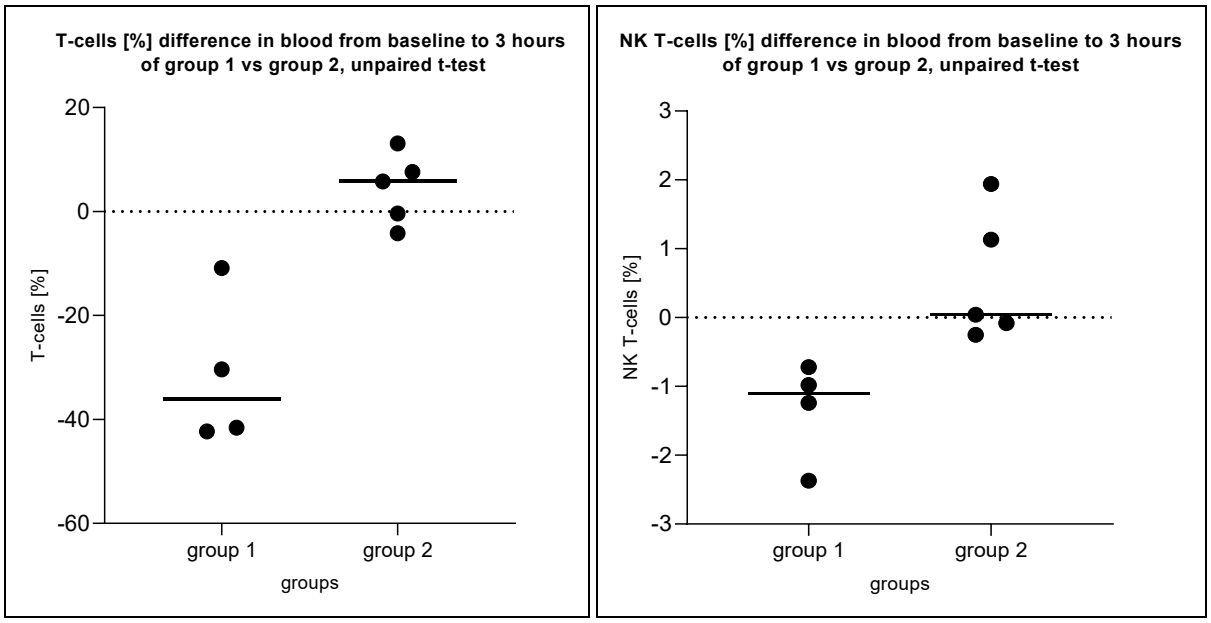


Figure 47 *(left)**:** Significant (p = 0.0018) difference between group 1 (n = 4) and group 2 (n = 5) in changes of T-cell populations and* ***Figure 48*** *(right)**:** Significant difference (p = 0.0136) in NK T-cell population in blood samples from baseline to 3 hours post dose using unpaired t-test.*

In both groups, group 1 and group 2, a significant decrease in lymphocytes in the blood could be measured between baseline and 3 hours (p = 0.0082 and p < 0.0001 respectively). Even a greater decrease is observed in group 2, the difference values between group 1 and group 2 in changes of the lymphocyte population in baseline and 3-hour blood samples are statistically non-significant (p = 0.1073) (**Figure 49**).

The B-cell population also showed a significant (p = 0.0213) drop in the comparison of the baseline to the 3-hour blood values of group 2, the lymph-cannulated animals. In contrast, in group 1, the non-operated animals, a significant (p = 0.0245) increase was seen. Accordingly, the difference of changes of the B-cell population in baseline and 3-hour blood samples between groups 1 and 2 is statistically significant (p = 0.0008) (**Figure 50**).

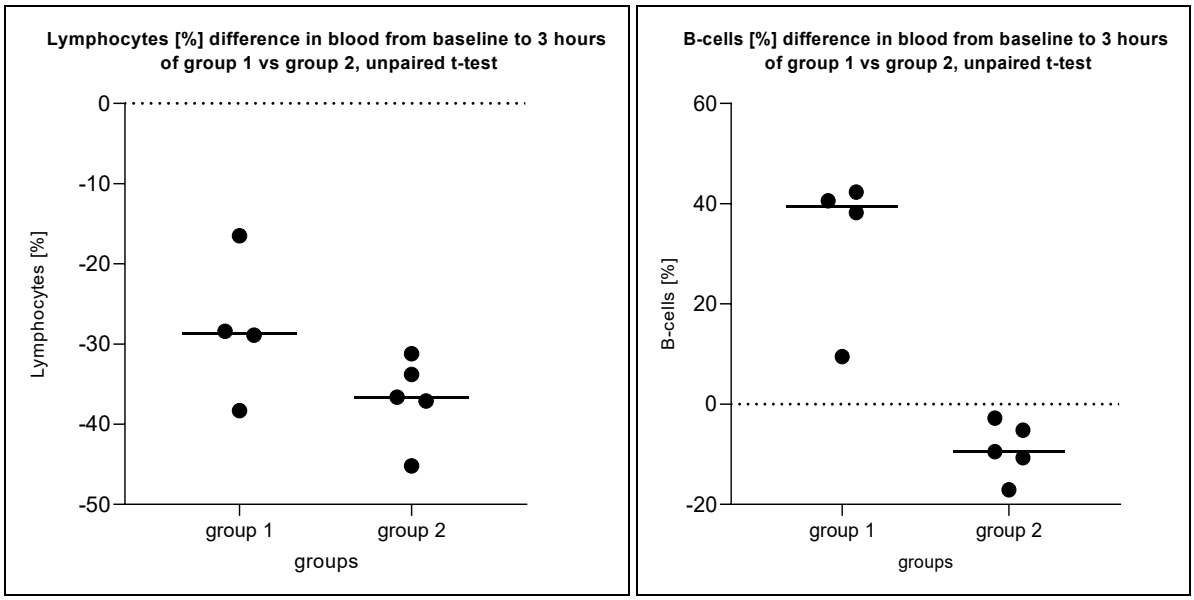


***Figure 49** (left): Non-significant (p = 0.1073) difference between group 1 (n = 4) and group 2 (n = 5) in decrease of lymphocyte population in blood samples from baseline to 3 hours post dose using unpaired t-test and **Figure 50** (right): Significant (p = 0.0008) difference between group 1 (n = 4) and group 2 (n = 5) in changes of B-cell population in blood samples from baseline to 3 hours post dose using unpaired t-test.*

Observed effects in lymph

Over the 3 hours collection period, a decrease in lymphocytes in the lymph fluid of group 2 can be shown (**Figure 51**). The statistical evaluation with the paired t-test showed that the difference of the lymphocyte populations between baseline and 3-hour samples is significant (p = 0.0086) (**Figure 52**).

A large cell population could be detected in the lymph samples of group 2 that could not be found in the blood samples of this group. An increase in this large cell population can be observed over the time course of 3 hours (**Figure 53**). When comparing the large cell population of baseline lymph samples with the large cell population of 3-hour lymph samples, a statistically significant increase can be evaluated (p = 0.0415) (**Figure 54**).

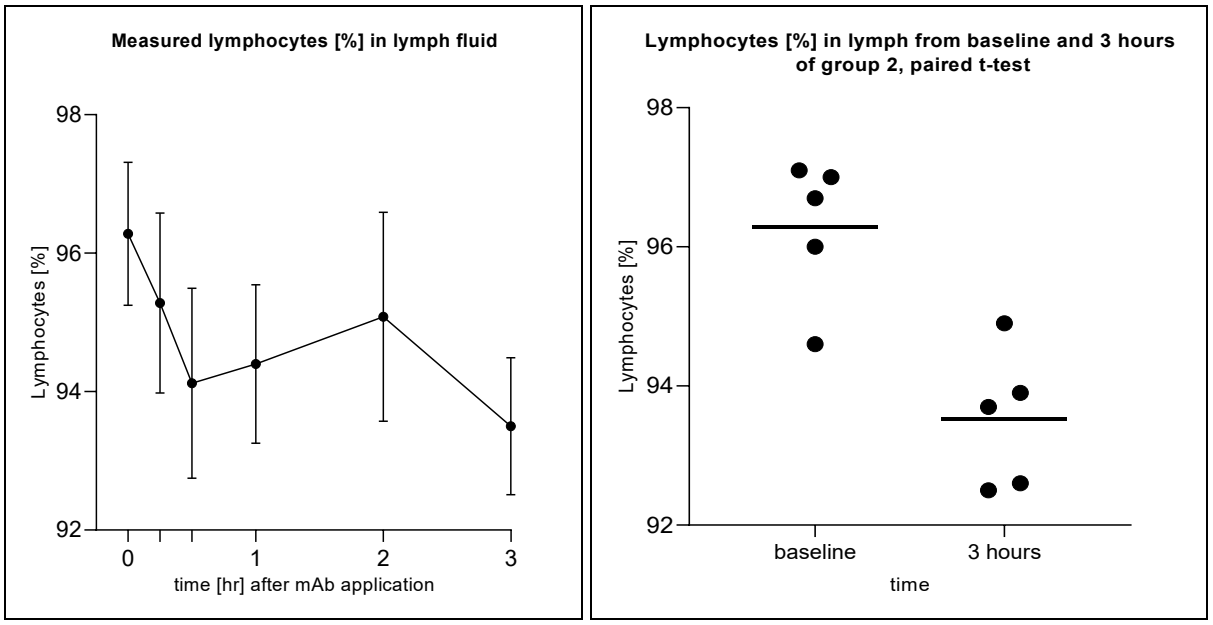


Figure 51 *(left): Mean number of lymphocytes [%] in lymph samples of group 2 (n = 5) over a time course of 3 hours after compound 4 administration and* ***Figure 52*** *(right): Significant (p = 0.0086) decrease of lymphocytes, comparing baseline lymph samples to 3 hours lymph samples of group 2 (n = 5).*

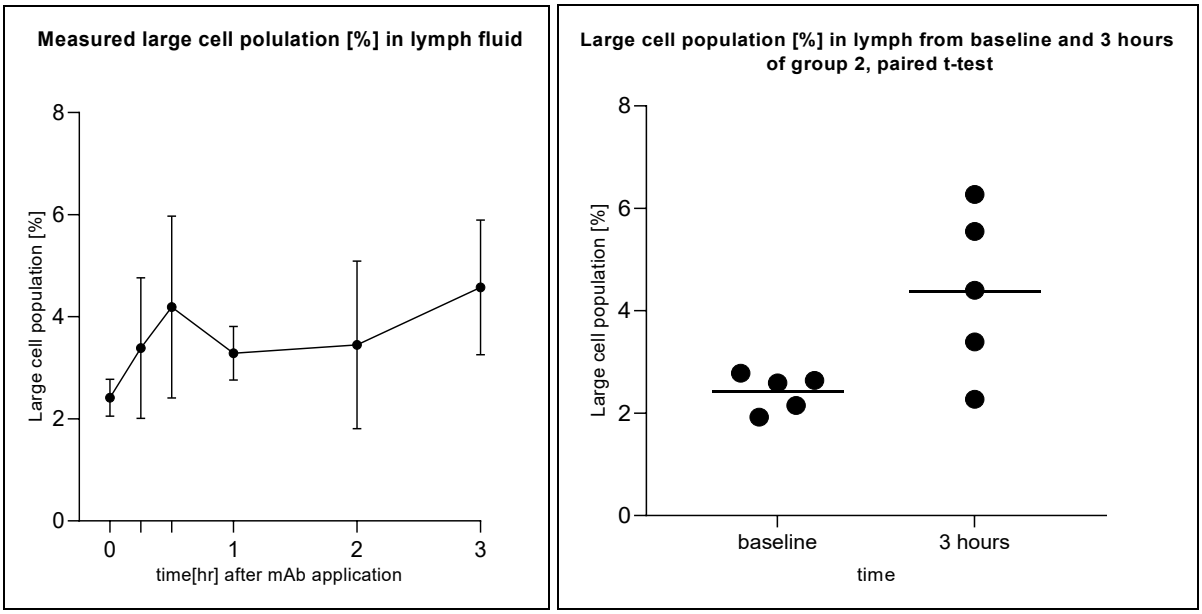


Figure 53 *(left): Mean number of large cell population [%] in lymph samples of group 2 (n = 5) over a time course of 3 hours after compound 4 administration and* ***Figure 54*** *(right): Significant (p = 0.0415) increase of the large cell population comparing baseline lymph samples to 3 hours lymph samples of group 2 (n = 5).*

VII. DISCUSSION

The aim of this thesis was the investigation of the lymphatic system with regards to the absorption and kinetics of orally administered, highly lipophilic drugs and, as a second aspect, the immunological processes after subcutaneous administration of large molecules. Four different tool compounds were used to verify the surgical model and to explore the uptake, modification and distribution of drugs within the lymphatic system as well as the release into the circulation. The results from this thesis can help to improve drug formulations to enable increased oral bioavailability, thus reducing pill burden or dose volume and potentially increasing patient compliance. Therefore, an *in-vivo* model including the cannulation of the thoracic duct in anesthetized rats was established which allows a direct measurement of concentrations and effects on cellular composition of the compounds in lymph fluid. The following questions were addressed to obtain an improved understanding of the role of the lymphatic system in drug delivery.

Question 1

Can an animal model for the collection of lymph be established?

The surgical method of thoracic duct cannulation and following lymph sample collection has been described in the literature in various publications for anesthetized and freely moving conscious rats, as well as for some larger animals such as pig, sheep, and dogs (Edwards *et al.*, 2001). Even though the dog model allows the oral administration of full-scale clinical human doses, the rat model enables the comparison to historical data such as biochemical processes of lipid absorption (Trevaskis *et al.*, 2005). Trevaskis *et al*. (2005) and Edwards *et al.* (2001) reported many advantages but also disadvantages of the anaesthetized rat model. On the one hand, anesthesia has as positive effect as it enables a constant lymph collection, since the animals cannot manipulate the cannula, on the other hand it has a negative impact on lymph flow rate, explained by a decrease in motility and function of the gastrointestinal tract (Dahan *et al.*, 2007): A threefold reduction of the lymph flow rate due to the influence of anesthesia is described by Dahan, Mendelman *et al.* (2007), corresponding to the report of Edwards *et al.* (2001). However, in the experiments described in the thesis involving the anesthetized rat model in terminal studies, it was possible to collect a comparable amount of lymph fluid over the entire collection period, enabling the measurements of concentrations and effects of the compounds in lymph fluid. In addition, the anesthetized rat model allowed to carry out the experiments at the same day, without surgical recovery time. Due to the challenging surgical method, a surgery-related failure of approximately 20 % must be taken into account during the experiments (Edwards *et al.*, 2001). Dahan *et al.* (2014) described a success of only up to 50 % in the freely moving conscious rat model. This suggests that a significantly higher failure rate can be expected in the awake rat model. Furthermore, performing the terminal experiment under anesthesia reduces the burden for the animals. Nevertheless, for a long

term measurement of the parameters it will be necessary - after establishing the surgical technique – to establish a model with freely moving conscious rats.

The importance of both flushing the catheter with heparin-saline solution and using a heparin-coated collection tube became clear during the experiment. The preparation of the catheter with heparin solution has already been described in some articles (Webb & Sanders, 1991; Wang *et al.*, 2012; Trevaskis *et al.*, 2015a). During the establishment of the surgical procedure in the context of this thesis, the unexpected coagulation of lymph fluid, when collected in serum Eppendorf tubes, repeatedly caused problems for sample analyses despite the use of heparinized saline, to flush the catheter. Ionac (2003) illustrated a detailed description of the surgical technique and lymph collection, using heparinized 10-mL Eppendorf tubes. Replacing the serum Eppendorf tube in the experiments with a Lithium-Heparin tube reduced the clotting of the lymph noticeably. Also Choo *et al.* (2014) reported the use of heparin tubes for his experiments (Choo *et al.*, 2014). Nevertheless, some animals had to be excluded from the statistical evaluation due to obstructed catheters during lymph collection. To prevent clotting in the catheter, the use of a silicone T-tube, assembled of two long arms that converge to a proximal end, could be an alternative to the simple polyethylene catheter. One of the long arms, also called “heparin line” is connected with a microinfusion pump, infusing the catheter with 1.4 mL/day heparinized saline solution (Girardet, 1975). Even if Girardet (1997) described the use of the T-tube catheter in long-term studies of up to 7 days, its use in the anesthetized rat model might also prove to be suitable. It needs to be emphasized that the material of the cannula has an important impact on the success of the procedure. While in these experiments, as well as Edwards *et al.* (2001), polyethylene catheters, which enable an accurate catheterization of the lymph vessel, were used, Ionac (2003) preferred the use of a silicone tube due to a better intraoperative handling. However, in the experiments of this thesis, correct handling of the polyethylene catheter was a “breakthrough” in establishing the surgical method of cannulating the lymphatic system. As also reported by Boyd *et al*. (2004), special attention should be paid to the preparation of the catheter. Using a bent wire, the end of the polyethylene catheter, which was to be introduced into the duct, was preshaped in boiling water. In addition, the tip of the catheter was cut with scissors, to achieve a beveled shape (Boyd *et al.*, 2004). During the operations in the experiments of this thesis, the forming of the catheter into an "L-shaped loop" facilitated the handling since it could be placed in the starting position before cannulation. This “L-shaped catheter” is better adapted to the anatomical course of the thoracic duct than a straight one and so prevents unvoluntary movements of the catheter and thus prevents damage to the lymphatic vessel. Furthermore, the preparation improved the attachment of the catheter to the lymphatic vessel, as it was less flexible and thus avoided a quick slip-out.

Another important detail in the choice of material is the use of black silk when attaching the catheter to the duct. Consistent with the literature, only silk was suitable, since the use of other sutures, such as vicryl, damaged the lymphatic vessel during knotting down the ligation

(Girardet, 1975; Li *et al.*, 2011; Trevaskis *et al.*, 2015a). Concerning the color, the use of black silk was preferred, because the contrast made identification next to the white thoracic duct much easier, thereby minimizing the risk of injuring the lymphatic vessel. In addition to the sutures, according to the description of Edwards *et al.* (2001), a drop of surgical glue to seal the entry point around the catheter while enhancing the cannula's retention was used (Edwards *et al.*, 2001). It was important to apply a small amount of drops, as the glue loses its flexibility when it dries out and can tear the surrounding tissue when the catheter is manipulated. Because of this side effect, a method of silk and glue combination was successfully developed.

For proving that cannulation of the thoracic duct in anesthetized rats has been successfully established, one male rat received an oral dose of compound 1, vitamin D_3, in olive oil. It is already known from the literature that the fat-soluble vitamin is very well absorbed by the lymph (Maislos *et al.*, 1981; Sitrin *et al.*, 1982). Since vitamin D_3 metabolism in literature was tested both, in the anesthetized and in the conscious rat model, no interactions with the anesthesia had to be expected (Dahan *et al.*, 2007). Reports of Maislos *et al.* (1981) determined an increase in concentration of vitamin D_3 in the lymph at the 120 minutes value, reaching a peak after a collection time frame of 150 to 180 minutes. While an increase in the concentration of vitamin D_3 could also be observed in the experiments of this thesis, no peak was reached within 300 minutes (**Figure 15**). The highest measured concentration of compound 1 was 520.7 ng/mL measured at the 5 hours value. Dahan *et al.* (2007) collected lymph over a period of 8 hours in anesthetized rats and were able to measure a peak after 5 hours. Since this experiment of the thesis was conducted to confirm the successful establishment the duration of the collecting time was limited to the 5 hours post dose. However, it was possible to show that vitamin D_3 in olive oil was absorbed by the lymph after oral administration and the model was suitable for the planned experiments.

Question 2

Are the orally administered compounds used here absorbed via the lymphatic system?

In the literature, it is well known, that the physicochemical characteristics of substances have an important influence on the site of absorption for example as described by Trevaskis *et al.* (2008). Lipinski *et al*. (1997) already defined with the ‘rule of 5’ (**Chapter Literature 2.2**) the physicochemical requirements of drugs with a good oral bioavailability (Lipinski *et al.*, 1997). Taking these conclusions into account, compounds 2 and 3 were selected for the experiments in this thesis with oral administration. Because it is believed that lymphatic absorption and transport is most relevant for highly lipophilic drugs, substances with high molecular weight, distribution coefficient log P and high lipophilicity were primarily considered (Trevaskis *et al.*, 2015b). In addition, the two substances are known to exhibit poor oral bioavailability. In order to improve the oral bioavailability for example to decrease a high pill burden in patients, it

was necessary to figure out whether the substances are absorbed via the lymphatic system. From both test compounds 2 and 3, concentrations could be measured in the lymphatic fluid, as it was already shown for compound 1 which was used to establish the model. Dependent on the formulation, significant differences between the measured concentrations were observed, which is discussed in detail in Question number 3.

One rule defined by Lipinski *et al*. (1997) states that the molecular weight must not exceed 500 Da to enable sufficient bioavailability. Although compounds 2 and 3, with 991.08 Da and 705.65 Da, respectively, have relatively high molecular weights for orally administered drugs (< 1000 Da, (Svatoš, 2010)), an impact of the molecular weight on lymphatic exposure was not seen. As reported by Reddy & Murthy (2002), a high molecular weight is an explanation for poor oral bioavailability, because of the limited permeability through the tight junctions of blood capillaries (Reddy & Murthy, 2002). This explains a better absorption by the 'cleft-like' intercellular junctions of the lymph capillaries, and thus the possibility of improving the oral bioavailability by promoting lymphatic absorption. Nevertheless, it does not preclude the uptake of smaller molecules, as demonstrated by the absorption of compound 1 which has a molecular weight of 384.64 Da. Porter & Charman (2001) described some small molecules, that are preferably taken up by the lymphatic pathway and not by the 500 times faster flowing blood, due to their at least 50.000 times higher affinity to the lymph lipoprotein (Porter & Charman, 2001). Also Ryšánek *et al.* (2020) were not able to demonstrate any correlation between intestinal lymphatic absorption and molecular weight (Ryšánek *et al.*, 2020).

Another physicochemical property that, according to Lipinski *et al.* (1997), influences oral bioavailability is the log P, which should not be less than 5. Other authors, such as Charman (2019), reported on the importance of lipid solubility and log P for lymphatic absorption as well as the impact on oral bioavailability (Charman, 2019). Also Ryšánek *et al*. (2020) described the poor lymphatic uptake of substances with a log P < 5 by comparing 103 clinically relevant drugs. They reported a lymphatic absorption of less than 3 % for substances with log P values < 4.8 (Ryšánek *et al.*, 2020). Based on this information, the compounds for the formulation experiments of this thesis were selected. In particular, high fat solubility and log P were properties that were required for the subsequent formulation question. The compounds used for the experiments both had a log P value over 5, for compound 2 it was 8.163 and for compound 3 it was 7.31. Also compound 1 (vitamin D_3), whose uptake into the lymphatics was already demonstrated by Sitrin *et al.* (1982) had a log P of 7.13 and met the requirements. Although no correlation between the log P and the high lipophilicity can be shown in the experiments of this thesis, all three compounds with a high lipophilicity and log P value > 5 were absorbed through the lymph (**Figure 15, 16 and 24**). In contrast to Myers & Stella (1992), who demonstrated in their experiments the surprisingly poor lymphatic uptake of penclomedine, a highly lipophilic drug with a log P value of 5.5, experiments in this thesis were consistent with the results of Charman (2019) (Myers & Stella, 1992).

Brocks & Davies (2018) described a relation between the lymphatic absorption of drugs and a high plasma protein binding. This is particularly important for compound 3, with a plasma protein binding of over 98 %. Drugs highly bound to plasma proteins also bind to lymph lipoproteins and are transported to the blood circulation via the lymphatic system (Brocks & Davies, 2018). Also Trevaskis *et al.* (2009) investigated the lymphatic uptake and food influence on oral bioavailability of a cannabinoid receptor agonist (CRA) with a plasma protein binding of > 99.5 %. Approximately 80 % are associated with plasma lipoproteins. This means, highly lipophilic drugs with a high plasma protein binding are transported via chylomicrons and additionally by binding to lipoproteins, primarily to very low-density lipoproteins (VLDL) (Trevaskis *et al.*, 2009). In line with the reports by Brocks & David (2018) that lipids also cause a transient increase in the binding of drugs to lipoproteins, the high plasma protein binding could also be a reason for the significantly better uptake of compound 3 into the lymph by help of the lipid based formulation (**Figure 24**).

Question 3

Can the oral bioavailability of orally administered compounds be improved by administration of lipid based formulations ?

Bergström *et al.* (1954) studied the intestinal fat absorption by testing the lymphatic absorption of oleic acids in the anesthetized thoracic duct cannulated rat model. They detected that 63 % of fed oleic acid were transported via the thoracic duct (Bergström *et al.*, 1954). Also Charman & Stella (1991) described the different properties of co-administered lipids such as chain length of fatty acids, degree of unsaturation, and rate of fatty acid uptake that are critical to either lymphatic or direct portal vein blood uptake. While fatty acids such as oleic acid, which is an unsaturated fatty acid with a chain length of 18 carbons (C18:1), are largely absorbed by the intestinal lymph, a large portion of short-chain (less than 12 carbons) fatty acids are taken up by the portal blood and therefore are unimportant for the chylomicron formation (Charman & Stella, 1991). Furthermore, when selecting the lipid formulation, attention must be paid to the structure of the lipids. By examining the effect of different lipids on the lymphatic absorption of Dichlorodiphenyltrichloroethane (DDT), Charman & Stella (1986) supported the assumption of differential effects from differently structured lipids on the rate and amount of drug transport. Fatty acids can be taken up directly by the intestinal epithelium, while triglycerides need to be first hydrolyzed into fatty acids (Charman & Stella, 1986). Taking this information into account, a lipid based formulation containing oleic acid was selected for the administration of compound 2 and compound 3 to improve the oral bioavailability and taking advantage of the high lipophilicity. The selected formulation was composed of 10 %PEG400: 10 % Cremophor EL: 80 % Oleic acid. It has already been used occasionally in toxicology studies for similar compounds, such as other Bcl-2 inhibitors. In order to be able to draw a direct comparison with conventionally used formulations, the compounds used for the experiments were also administered in a non-lipid based formulation.

This comparison formulation is standardly used in internal toxicology and DMPK studies and is constituted of 10 % DMSO: 90 % PEG400. Comparing the mean AUC difference between the lipid based and non-lipid based formulation groups, compound 2 showed a statistically significant increase in oral bioavailability with the lipid based formulation in lymph-cannulated groups ($p = 0.0325$) and plasma satellite groups ($p = 0.0297$) (**Figure 22, 23 and Table 15**). Also for compound 3, a statistically significant increase in oral bioavailability due to the lipid based formulation was shown in lymph-cannulated groups ($p = 0.0179$) and in the plasma of the corresponding satellite groups as well, $p = 0.0046$ (**Figure 31, 32 and Table 20**). These results are consistent with the literature such as Charman & Stella (1986 and 1991) and Bergström *et al.* (1954). All these groups could show that a formulation composed primarily of oleic acid acts as a promoter for the lymphatic absorption of highly lipophilic drugs and thus also has a positive effect on oral bioavailability. Moreover, Van Greevenbroek *et al.* (1996) promoted this theory with the results of their *in-vitro* experiments. They investigated the effect of different fatty acids on lipoprotein synthesis using a colorectal adenocarcinoma cell line 2 (Caco-2) *in-vitro* model. Caco-2 cell lines are human colon carcinoma cells whose differentiation take place while cultured into enterocytes. After incubating various fatty acids on the Caco-2 model, they were able to measure the highest levels of triglyceride and lipoprotein formation in cells that were incubated with unsaturated oleic acid (van Greevenbroek *et al.*, 1996).

In such a Caco-2 model, Krishna *et al.* (2001) investigated the permeability of 2 highly lipophilic Schering compounds, SCH-A and SCH-B, in combination with DMSO. Although Marren (2011) described improved penetration in transdermal administration of hydrophilic and lipophilic drugs with DMSO (Marren, 2011), Krishna *et al.* (2001) could not confirm this effect of DMSO for the normally orally administered SCH-A and SCH-B drugs. For both compounds the poor permeability resulted in a very low absorption (Krishna *et al.*, 2001), leading to believe that the 10 % DMSO used non-lipid based formulation in the experiments of this thesis had no influence on the lymphatic absorption.

Another ingredient of the non-lipid based formulation used in the experiments of this thesis is 90 % PEG400. Because poor water solubility of drugs is one of the limiting factors on the systemic exposure, PEG400 is a commonly used co-solvent for such hydrophobic candidates (Ma *et al.*, 2017). Ma *et al.* (2017) examined the influence of PEG400 on the systemic exposure of oral drugs. Beside effects on permeability, drug metabolizing enzymes, transporters and gastrointestinal transit time, they assumed an influence on the lymphatic transport due to an increase of the lipid solubility (Ma *et al.*, 2017). Taking this assumption into account, an even clearer result could be expected in the experiments of this thesis when a completely aqueous solution would have been used.

However, it was possible to demonstrate the essential role of the lipid based formulation for highly lipophilic drugs with poor oral bioavailability such as compounds 2 and 3. This finding is

of great importance, especially in the case of drugs used for the treatment of cancer like Bcl-2 inhibitors because an improved formulation could reduce the sometimes very high pill burden. An additional advantage of supporting the lymphatic uptake is the bypass of the hepatic first pass effect and the associated direct metabolism by the cytochrome P450 enzyme family (Elz *et al.*, 2021). This is also particularly important for Bcl-2 inhibitors such as compound 2, since reports like that of Agarwal *et al.* (2017) showed that the enzyme cytochrome P450 plays a major role in the elimination of Bcl-2 inhibitors (Agarwal *et al.*, 2017). For other substances, e.g. antifungal agents such as compound 3, the first pass effect, leads to formation of the active metabolite Hydroxy-Itraconazole (Heykants *et al.*, 1989). As Heykants *et al.* (1989) assumed a similar potency of Hydroxy-Itraconazole as for Itraconazole itself they added up the AUCs of both active substances for calculating the absolute oral bioavailability of 80 %. Taking this assumption into account, a further experiment with compound 3 could also include the measurement of Hydroxy-Itraconazole.

Question 4

Has olive oil administration an effect on the oral bioavailability?

Rats undergoing surgery to cannulate the lymphatic system received 0.1 mL of olive oil by gavage 1.5-2 hours before compound administration with the purpose of improving visualization of the duct. The oil produced a milky white coloration of the normally transparent lymphatic system and thus enabled identification of the lymphatic system. In order to be able to assess the effect of olive oil on the oral bioavailability of compounds used in this thesis, two different satellite groups were dosed with or without oil pretreatment for the experiments. Charman & Stella (1986) researched the lymphatic transport of benzo(a)pyrene in 50 µmol or 500 µmol of olive oil and of DDT in 50 µl or 200 µl of a lipid vehicle. Even though the administered amount of lipid vehicle had no effect on lymphatic absorption, higher concentrations of DDT per chylomicron in 50 µl lipid vehicle were measured, leading to the assumption that chylomicron transport can reach a saturation (Charman & Stella, 1986). Comparing the two groups of satellites that received the lipid based formulation either with or without previous olive oil administration, a similar graph progression was found for compound 2 with no significant difference between the groups (**Figure 20**). Comparing the two groups of compound 3 higher mean concentration levels in plasma were measured in the satellite group that received only the lipid based formulation, compared to the group that was administered with olive oil first (**Figure 28**). The evaluation of the AUC's confirmed a statistically significant decrease in the oral bioavailability by administering olive oil before the treatment with compound 3 in the lipid based formulation (**Figure 29 and Table 18**). These results show that olive oil has no enhancing effect on lymphatic absorption. In contrary, it is particularly clear from the results of compound 3 that the administration of oil has a negative impact on the effect of the lipid based formulation. These results are consistent with the assumption of Charman & Stella (1986) about a saturation in chylomicron formation. It is

assumed that it is not the volume but the time-delay of 1.5-2 hours between the administration of the olive oil and the compound in the lipid formulation that is an important aspect on the level of chylomicrons formation. However, olive oil is essential to facilitate cannulation of the lymphatic system and thus enables successful cannulation. Also the use of a lipophilic dye such as Sudan black as described by Edwards *et al.* (2001) in a pig model has comparable influences on the experiment as olive oil (Edwards *et al.*, 2001).

An additional effect possibly explaining the decrease in oral bioavailability is the gastric emptying that is slowed down by oral administration of oil. By comparing the gastric emptying of different lipids, using indirect markers, Mu & Porsgaard (2005) demonstrated a very fast emptying starting after 15 minutes and reaching a maximum after 45-60 minutes after oil administration. Since high concentrations of the marker substance could still be measured after three hours, it was concluded that after an initial rapid gastric emptying, the lipolysis products have an inhibiting effect on further gastric emptying (Mu & Porsgaard, 2005). Considering the results of Mu & Porsgaard (2005), it is possible that in the experiments of this thesis the olive oil administration before the surgeries already inhibits gastric emptying. In conclusion, the low increase and slow decrease in concentrations observed in compound 2 given to the satellite group that received both, the oil and lipid formulations could be a consequence of the inhibited gastric emptying by the olive oil, which is prolonged with the administration of the lipid formulation (**Figure 20**). Therefore the planned long term experiments in freely moving conscious rats could avoid this possible oil effect.
A weaker increase in concentrations can also be observed for compound 3. Because there is no peak observed within the six hours of sample collection, the peak and anticipated drop in concentration could not be assessed (**Figure 28**). This consideration would be in line with the results of Noguchi *et al.* (1985) who showed a significant delay in lymphatic transport of the tested drug in 500 µl oleic acid compared to the formulation in only 200 µl oleic acid (Noguchi *et al.*, 1985). In the same article the result was explained by the “prolonged gastric emptying time and/or the saturation of a process(es) involved in the lymphatic transport of lipophilic molecules (…)” (Noguchi *et al.*, 1985). However, this assumption requires further investigations.

In contrast to the significant mean plasma AUC difference between the groups receiving only the lipid based or non-lipid based formulation (**Figure 23 and Table 16**), there is no significant difference between the plasma AUCs of the satellite animals receiving the non-lipid based formulation with olive oil and the lipid based without olive oil (**Figure 21 and Table 15**). This indicates that the pre-administered olive oil has an enhancing effect on oral bioavailability of compound 2 in animals receiving the non-lipid based formulation. Lipids transported from the stomach into the duodenum stimulate the secretion of bile and pancreatic fluids, which play an important role in the formation of chylomicrons (Yáñez *et al.*, 2011). Holm *et al.* (2012) reported the importance of bile salts after observing significantly reduced halofantrine concentrations in the lymph in bile duct cannulated rats (Holm *et al.*, 2012). It is assumed that

similar to the described food effects (Yáñez *et al.*, 2011), olive oil promotes the formation and release of bile salts and pancreatic fluids and thereby improves the oral bioavailability of non-lipid based formulations.

The results of compound 3 concur with the well-known problem of inconsistent absorption, as also described by other authors, e.g. De Beule (1996), Grant & Clissold (1989) and Lestner & Hope (2013) (**Figure 25**). Van Peer *et al.* (1989) found an increase in concentrations of Itraconazole when taken immediately after a unspecified meal (Van Peer *et al.*, 1989). In the experiments of this thesis no enhancing effect by the olive oil could be seen in rats. With the exception of the outlier, a later increase in concentrations was initially observed, compared to the animals receiving the non-lipid based formulation. Only after five hours was there a higher C_{max} measured in the group that had received olive oil before dosing.

However, the results of this thesis show that the olive oil has a similar bioavailability enhancing effect on the tested compounds as the lipid formulation. This could be shown by comparing the AUCs of animal groups that received the non-lipid based formulation after olive oil administration, to the groups that received only the lipid based formulation (**Figure 21**) which did not result in a statistical difference.

Along with the assumption of the limiting factor of olive oil on the lipid based formulation the results indicate that highly fat-soluble drugs are better administered in a lipid based formulation in the fasted state than in non-lipid based formulations immediately after meals. Due to symptoms of e.g. malignancies which are often treated with substances like compound 2, regular food intake is not possible in some patients. Moreover, the intake of diets containing a high degree of fatty substances cannot be guaranteed. In order to ensure a constant and regular treatment, a change of formulation would be a logical consequence. The success of therapy and making treatment more convenient and tolerable for patients are important and current issues in oral medication development. This is shown by formulation approaches such as amorphous solid dispersions (ASD) which are standardly used in the pharmaceutical industry, mainly to improve the oral bioavailability of poorly water-soluble drugs (AboulFotouh *et al.*, 2020). The results of the experiments demonstrate the importance of the intestinal lymphatic absorption pathway for highly lipophilic drugs such as compound 2 and 3. Therefore it is inevitable to pay more attention to lipid based formulations, rich in triglycerides, like vehicle 2 (10 % PEG400: 10 % Cremophor EL: 80 % Oleic acid*),* to improve oral bioavailability and thus avoid high pill burden.

Question 5

Is Fluorescence Activated Cell Sorting (FACS) analysis suitable for the analysis of lymphatic fluid?

The analysis of cells in lymph nodes and other organs of the immune system by FACS has been described frequently in the literature (Carter *et al.*, 1983; Harris *et al.*, 2002; Noh *et al.*, 2008). In contrast, very little information is available on the application of FACS analysis for the measurement of the cellular composition of lymph fluid: Three publications described this in sheep, one in pediatric children and one in rats (Bonneau *et al.*, 2006; Trevaskis *et al.*, 2010; De Veer *et al.*, 2012; Neeland *et al.*, 2014; Foster *et al.*, 2020). This is due to the fact that collection of lymph fluid is a very demanding surgical method that requires highly skilled personnel (Chaudhary *et al.*, 2014). Antibodies are important drugs for the therapy of immune-mediated diseases (e.g. multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease) or cancer diseases (e.g. leukemia, breast cancer, colorectal carcinoma (Lu *et al.*, 2020). Because of denaturation processes of proteins in acidic environment, oral administration of protein-based therapeutic agents, including monoclonal antibodies, is not possible (Lin, 2009). Therefore, they are usually administered intravenously or subcutaneously. Subcutaneous administration is preferred because it is more cost-effective in addition to higher patient convenience (Rahimi *et al.*, 2022). It is assumed that the lymphatic absorption also plays a key role in the absorption of subcutaneously administered large molecular bio-therapeutics. Because of that, it is of great importance to understand the cellular composition of lymph fluid for potential immunological reactions, such as antidrug antibody (ADA) formation within the lymph (McLennan *et al.*, 2005; Rahimi *et al.*, 2022). Immunophenotyping of blood samples by FACS analysis is a standardly used method in the clinic for routine diagnostics, such as hematology and immunology (Roederer, 2002). Because the very few available reports on this topic are additionally quite old, in this thesis the analysis of lymph fluid of rats with the BD FACSVerse^{TM} Cell Analyzer, BD Biosciences in the pilot study with compound 4 was established. The baseline lymph samples were used to determine the cellular composition of the lymph fluid.

Consistent with the reports of Morris & Courtice (1977) who defined the main cellular component of thoracic lymph fluid in sheep as being 95 % small lymphocytes, the baseline samples of the study with compound 4 (of this thesis) contained an average of 96.28 % lymphocytes in the lymph fluid of rats. The lymphocytes measured were also found to be significantly smaller (on average 16.00 % smaller) than those normally found in blood (**Figure 36 and 37**). Azargoschasb (1963), who primarily described the cell types occurring in the thoracic lymph fluid of rats qualitatively and only partially focused on the quantitative relationships, was also able to determine a high proportion of small lymphocytes by using an electron microscope. Both authors, Morris & Courtice (1977) as well as Azargoschasb (1963), described the microscopically visible size difference between small lymphocytes and large

lymphocytes in lymph. Reinhardt & Yoffey (1957), who studied the lymphatic cellular composition of guinea pigs, noted such a difference in lymphocyte size as well (Reinhardt & Yoffey, 1957). When counting the differences microscopically, Reinhardt & Yoffey (1957) found that large lymphocytes make up on average 2.70 % of cells in the lymph.

While Azargoschasb (1963) made no statement about the proportion of large lymphocytes in rats' lymph fluid, Morris & Courtice (1977) were able to measure 5 % of large lymphocytes in the lymphatic fluid of sheep. In contrast to the results of Morris & Courtice (1977), but in accordance with Reinhardt & Yoffey (1957) in the experiment of this thesis an average of 2.42 % of a large cell population was detected. It is assumed that this large cell population consists of T- and B-lymphocytes since they are either $CD45R^+$, $CD3^+$ or $CD4^+$. The difference to the results of Morris & Courtice (1977) might be explained by a species difference in lymph composition.

Lymphocytes arise from the bone marrow, thymus and extrathymic tissue (Shimizu, Sugahara et al. 1999). After differentiation and mature processes naïve lymphocytes are in constant movement between blood circulation and lymphatic system. The cells are able to leave the blood stream, to migrate into the tissue and to return back into the blood circulation with the lymph fluid, passing the thoracic duct (Ford & Simmonds, 1972). The high proportion of lymphocytes in the lymph could be explained by this ability of lymphocytes to leave the blood circulation by passing across the vascular endothelial cells (Schoefl, 1972). The fact that erythrocytes do not leave the blood circulation (Rovenská & Rovensky, 2011) supports the results of this thesis that no erythrocytes were found in the lymph samples. Additionally, it corresponds to the visual impression of very clean milky-white lymph samples. In contrast, Ross *et al.* (1952) reported an evident increase of erythrocytes in dog and rat lymph after ionized irradiation. Furthermore, they described zero to two erythrocytes per 100 white blood cells in normal lymph (Ross *et al.*, 1952). In the experiments of this thesis only minor bleeding occurred during surgery and these were immediately absorbed with swabs. The question arises whether the erythrocytes in normal lymph that were described in the results of Ross *et al.* (1952) can be explained by bleeding during surgery. Also, the worker group of Ross *et al.*(1952) itself made this hypothesis: *"…that entry of erythrocytes into the lymph system occurs even when there is no gross evidence of a hemorrhagic tendency."* This would also confirm the assumption that minor tissue pieces, caused by the surgical manipulation, obstructed the heparinized catheter in the animal that had to be excluded from the study (**Appendix, Section 5**).

Similar to lymphocytes, monocytes and granulocytes - belonging to the non-specific immune system – also have the ability of migrating into tissue (Leliefeld *et al.*, 2015). After reaching the affected tissue, granulocytes as well as monocytes phagocytose pathogens (Kantari *et al.*, 2008). Hampton & Chtanova (2019) reported an additional smaller portion of granulocytes reaching the lymph nodes of the inflamed tissue via the lymphatic vessels (Hampton &

Chtanova, 2019). Such a migration was also described for monocytes by Bonneau *et al.* (2006): after they injected latex microspheres intradermally, monocytes increased by 25 % in the lymph nodes. A very small amount of granulocytes (on average 0.05 %) and a very small population of monocytes (on average 0.17 %) were analyzed in the thoracic lymph samples of this thesis, consistent with the report of De Veer *et al.* (2012). This means that in addition to a significant majority of lymphocytes, that monocytes and granulocytes also form a small proportion of the lymph. With the detection of the different cell populations and subpopulations, it could be shown that the FACS analysis is a suitable tool for the diagnostic analysis of lymphatic fluid. Moreover, it might play a key role in better understanding of immunological processes.

Question 6

Is the subcutaneously administered immunology compound absorbed and distributed via the lymphatic system?

Immunotherapy is an essential clinical tool in the treatment of autoimmune diseases, cancer and organ transplants (Getts *et al.*, 2011). In addition, prophylactic treatments that strengthen the healthy immune system, such as vaccinations against yellow fever, play one of the most important roles in preventive healthcare (Terrie, 2010). Subcutaneous injections might be self-administered by patients, as opposed to intravenous injections (Rahimi *et al.*, 2022). In addition this route enables less frequent hospitalizations, thus it is also more cost-effective (Bittner *et al.*, 2018). Therefore, it is important to understand the dynamics of the transit from the subcutis into the circulation, as well as the interactions of the administered molecules with the immune system.

Cell populations in blood

Jones *et al.* (2017) put the molecular size of the administered protein and the absorption path after subcutaneous administration into context (Jones *et al.*, 2017). They determined that drugs with a molecular weight of < 20 kDa are taken up directly by the blood stream, while larger molecules with a weight of > 20 kDa are absorbed indirectly and then transported via the lymphatic system. Also Supersaxo *et al.* (1990) reported that molecules with molecular weight of > 16 kDa are prone to be absorbed via the lymph (Supersaxo *et al.*, 1990). This would mean that compound 4, mAb R73, with a molecular weight of 34 kDa, used in the pilot study of this thesis, is taken up via the lymphatic capillaries at the site of application. This monoclonal antibody (compound 4) activates T-cells by binding to the α/β TCR and leads to T-cell depletion in the blood circulation (Siemionow *et al.*, 2002). In concordance with the report of Siemionow *et al.* (2002), a significant decrease in T-cells and NK T-cells could be seen in the blood samples of group 1 (not lymph cannulated) over 3 hours, which confirms T-cell activation by the administered antibody. In contrast, such a decrease in T-cells and NK T-cells

was not detectable in the blood samples over the time frame of 3 hours of group 2 animals that had been lymph cannulated. Since the continuity between the lymphatic system and the blood circulation is interrupted by the catheterization of the thoracic duct, lymph is no longer transferred back to the blood. This means that the antibody is absorbed by the lymph and likely cannot be transferred to the blood stream. This may explain the lack of T-cell activation and thus the lack of decrease in the T-cell and NK T-cell populations observed in the blood samples of animals of the lymph cannulated group 2. Furthermore, this is consistent with the finding of Jones *et al.* (2017) and Supersaxo *et al.* (1990) that molecular weight has an impact on the absorption pathway, as it shows that mAb R73 is indeed taken up by the lymph and not directly by the blood stream. In addition, it is possible that administration of this antibody has an indirect effect on the B-cell population. While a significant decrease of 31.29 % on average in the T-cell population was measured in the 3-hour blood values of group 1, a significant increase in the B-cell population of 32.66 % on average was measured at the same time. Due to the almost identical ratio of increase and decrease of the two populations, it can be assumed that the B-cell increase is relative to the T-cell decrease. Also Dahlke *et al.* (2002) were able to show such a relative B-cell increase after a single dose of an Anti-RT7, a monoclonal antibody that causes massive T-cell depletion (Dahlke *et al.*, 2002).

In group 1, the absolute decrease in the number of total lymphocytes (an average decrease of 28.03 %) corresponds to the decrease in T-cells (an average decrease of 31.29 %). Because of this proportional decrease, corresponding to the antibody's mode of action, it is assumed that the lymphocyte depletion in group 1 is an effect of compound 4. In group 2 an even clearer decrease in lymphocytes can be observed. This could be a consequence of lymph cannulation. Rovenská & Rovensky (2011) reported on the recirculation of lymphocytes into the blood stream. Results from the experiments of this thesis demonstrated that lymph fluid contains an average of 96.28 % lymphocytes. Due to the interruption of the lymph-blood connection due to the catheterizing of the thoracic duct in group 2 animals, the recirculation described by Rovenská & Rovensky (2011) is anatomically not possible any longer. A consequence is the significant drop in lymphocytes in group 2 animals.

Cell populations in lymph

Besides the effects on cell populations in the blood, alterations in the cell population of lymph were evaluated, too. As already mentioned in question 5, the large cell population, which is thought to be large lymphocytes, showed a significant increase in the lymph. Yusuf & Fruman (2003) explained that quiescent (non-activated) naïve lymphocytes have reduced metabolism and cell size. In contrast, activated lymphocytes are larger due to a high rate of protein synthesis (Yusuf & Fruman, 2003). Taking this statement into account, these large cells could be activated lymphocytes. The increase of this cell population could be a result of the T-cell activation, caused by the administration and subsequent lymphatic absorption of the antibody. Due to the application site of the antibody which was in the subcutis of the hind legs

below the knee, it can be assumed that it passes several lymph nodes on its way to the thoracic duct. According to the description of Tilney (1970) the application site at the hindleg below the knee is drained by lymph vessels leading to the popliteal lymph node. Before this main efferent lymph of popliteal node flows into the cisterna chyli of the thoracic duct, it passes thorough iliac nodes and renal nodes (Tilney, 1970). The antibody could already have a first T-cell activating effect on the lymphocytes located in the lymph nodes. This results in an increase of the large cell population. This hypothesis is additionally supported by the proportionally significant decrease in total lymphocytes within lymph. However, due to the small number of cells in the large cell population, further diagnostic measurements must be taken in order to be able to prove this claim. For example, identification of markers that can clearly stain the large, activated lymphocytes could be a way to further explore this.

Outlook:

The results of this work show that both orally administered highly fat soluble small molecules and subcutaneously administered large molecules are absorbed via the lymphatic system and transported into the blood stream. The establishment of the surgical method of cannulating the thoracic duct in anesthetized rats made it possible to evaluate the lymphatic uptake of compounds whose limited oral bioavailability pose a challenge in terms of formulation. In order to circumvent surgery related side effects such as the restricted mobility of the animals or the effect on slower gastric emptying, it is planned to establish the method in a model with freely moving conscious animals. In addition to determining the pharmacokinetic properties of the compounds over a longer time period, an *in-vivo* model will also enable avoidance of the possible influence of olive oil, essential for visualizing the lymphatic system during surgery.

Furthermore, the pilot study with the subcutaneously administered antibody has shown that the lymphatic system plays an fundamental role in the uptake of such large molecules and is involved in immunological processes. Moreover, it was shown that the BD FACSVerse™ Cell Analyzer, BD Biosciences is not only suitable for the analysis of blood samples, but also for the analysis of lymph fluid samples. In further experiments, one goal will be a precise definition and analysis of the large cell population in the lymph fluid. Specific markers need to be identified to enable staining of the cells and thus, the determination of the cell population. Further studies are also planned to include an additional control group of operated animals in which the lymphatic system is cannulated but where no substance is administered. Such a control group was not used in the pilot study of this thesis because an anesthesia related influence in published reports was described as being low (Reinhardt & Yoffey, 1957), and it was not judged as justified in order to keep the number of animals low according to the 3R principle. Nevertheless, a control group must be considered for further experiments to rule out a possible surgery or anesthesia-related influence on immunological parameters such as the changes observed in immune cell populations. Another topic for the use of this model in further experiments is to investigate the dynamics of the interaction of large molecules with

the immune system in lymph nodes after a second administration (sensibilization). This should provide information about potential antibody drug complex formations as well as intolerance reactions, which often occur 15-30 minutes after repeated subcutaneous antibody administration. For a better understanding of this and other post-dose reactions, the cytokine releases in the subcutis, lymph nodes and lymph fluid should be analyzed in addition to the cell-analysis in the lymph. In conclusion, all of these examples and the results of the experiments of this thesis show the versatile possibility of using the model in different research areas.

VIII. SUMMARY

Oral and subcutaneous administration are the most commonly used routes of administration of pharmaceutics, since they are considered to be the safest form of drug delivery in addition to their cost-effective, patient-friendly handling. Nevertheless, oral bioavailability is limited by the physicochemical properties such as high molecular weight, low aqueous solubility and high lipophilicity (high log P). Also, the absorption and associated immune responses of subcutaneously administered large molecules are not completely understood, despite their extensive clinical use. Particularly in preclinical safety studies, the nature of side effects that occur after the second administration of a drug that was initially well tolerated, are not completely understood and can prevent further development of the drug. Determining the cellular composition of lymphatic fluid and enhancing targeted lymphatic absorption can improve understanding of immunological processes as well as oral and subcutaneous bioavailability.

For investigation of lymphatic uptake and distribution of orally and subcutaneously administered drugs, a surgical method of cannulating the thoracic duct in male rats was established. In first experiments animals received an oral single dose of two different highly lipophilic compounds in a non-lipid or lipid vehicle. For analyzing concentrations, lymphatic fluid was collected until 6 hours after dosing. Due to the surgery related influence on the systemic uptake, satellite animals were used to measure the systemic uptake into plasma. The results showed that the pharmacokinetic properties of both compounds meet the requirements for lymphatic uptake. Rats treated with the lipid formulation demonstrated 6- or 11- fold higher concentrations of compounds in the lymphatic fluid compared to non-lipid formulation. This improvement of the lymphatic uptake resulted in a significant increase of the bioavailability of compounds.

Additionally, a pilot study was conducted in which rats were injected subcutaneously with a T-cell activating antibody. Lymph and blood samples, collected in two different groups for up to 3 hours after compound administration were analyzed by the Fluorescence Activated Cell Sorting (FACS) analysis to evaluate the cellular compositions and the absorption pathway of the antibody. Besides the fact that lymphocytes were shown to be a main component of the lymph, making up on average 96.28 % of the cell population, it could be shown that the subcutaneously administered antibody was absorbed by the lymph. While significant T-cell activation was observed in non-operated animals, such an effect was not measurable in lymph cannulated animals, suggesting that the cannulation prevented the antibody from reaching the systemic circulation.

In conclusion, a surgical model for investigating the role of the lymphatic system in the absorption of orally and subcutaneously administered drugs was successfully established. It was shown that oral bioavailability can be improved by lipid based formulations and that the indirect absorption route should gain more attention in formulation development.

Furthermore, the establishment of the FACS analysis of lymph fluid paves the way for a better understanding of immunological processes after subcutaneous administration and can be used to further investigate the dynamics of the interaction of large molecules with the immune system.

IX. ZUSAMMENFASSUNG

Die orale und subkutane Applikation gehören zu den am häufigsten angewandten Darreichungsformen für Arzneimittel. Sie sind in ihrer Handhabung patientenfreundlich, ihre Herstellung und Anwendung ist kostengünstig im Vergleich zu intravenös verabreichten Medikamenten, und zudem gelten sie als besonders sicher. Die orale Bioverfügbarkeit ist allerdings durch physikalisch-chemische Eigenschaften wie hohes Molekulargewicht, hohen log P und geringe Wasserlöslichkeit beziehungsweise hohe Lipophilie limitiert. Auch die Absorption und die damit verbundenen Immunantworten von subkutan applizierten, großen Molekülen sind trotz ihres standardmäßigen Einsatzes nicht vollständig verstanden. Im Rahmen der Entwicklung von Human- und Veterinärarzneimitteln treten beispielsweise nach der zweiten Dosis eines zunächst gut vertragenen Medikaments oftmals unerklärliche Nebenwirkungen auf, die die Weiterentwicklung gefährden können. Aus diesem Grund sollen in dieser Arbeit die zelluläre Zusammensetzung von Lymphe analysiert werden, um ein besseres Verständnis immunologischer Prozesse zu ermöglichen. Zudem sollen durch die Untersuchung der lymphatischen Absorption Möglichkeiten identifiziert werden, die orale und subkutane Bioverfügbarkeit positiv beeinflussen.

Für die Untersuchung der lymphatischen Aufnahme und Verteilung von oral und subkutan applizierten Arzneimitteln wurde ein chirurgisches Verfahren zur Kanülierung des *Ductus thoracicus* bei männlichen Ratten etabliert. In ersten Experimenten erhielten die Tiere eine orale Einzeldosis von zwei verschiedenen hoch lipophilen Substanzen in einer nicht-lipid- oder in einer lipid-basierten Formulierung. Zur Analyse der Konzentrationen wurde Lymphflüssigkeit über einen Zeitraum von bis zu 6 Stunden nach der Substanzgabe gesammelt. Wegen des chirurgischen Einflusses auf die systemische Absorption wurden zusätzlich Satellitentiere verwendet, um die systemische Aufnahme ins Plasma zu messen. Die Ergebnisse zeigten, dass beide Substanzen über die Lymphe aufgenommen werden, wie es anhand ihrer pharmakokinetischen Eigenschaften erwartet war. Ratten, die mit der Lipidformulierung behandelt wurden, zeigten eine 6- oder 11-fach Konzentrationserhöhung der Substanzen in der Lymphflüssigkeit, im Vergleich zu Ratten, die die nicht-lipid basierte Formulierung erhalten hatten. Diese Verbesserung der lymphatischen Aufnahme führte zu einer signifikanten Erhöhung der Bioverfügbarkeit.

In einem zweiten Teil der Arbeit wurde eine Pilotstudie in Ratten durchgeführt, denen subkutan ein T-Zell-aktivierender Antikörper injiziert wurde. Anschließend wurden Lymph- und Blutproben über einen Zeitraum von 3 Stunden nach Substanzapplikation in zwei unterschiedlichen Gruppen gesammelt. Die Probenanalyse mittels Fluoreszenz-aktivierter Zellsortierung (FACS), sollte Aufschluss über die zelluläre Zusammensetzung der Lymphe geben und durch den Antikörper induzierte potenzielle Veränderungen zeigen. Neben dem Ergebnis, dass durchschnittlich Lymphozyten mit einem Anteil von 96,28 % einen Hauptbestandteil der Lymphe bilden, konnte gezeigt werden, dass der subkutan applizierte Antikörper von der Lymphe aufgenommen wurde. Während bei nicht operierten Tieren eine

signifikante T-Zell-Aktivierung im Blut festgestellt werden konnte, blieb ein solcher Effekt in der Gruppe der Lymph-kanülierten Tieren aus, was zeigt, dass der Antikörper durch die Operation die Blutzirkulation nicht erreichen konnte.

Ein chirurgisches Modell mit dem die Rolle des lymphatischen Systems bei der Absorption von oral und subkutan applizierten Medikamenten untersucht werden kann, konnte im Rahmen dieser Arbeit erfolgreich etabliert werden. Mithilfe dieses Modells konnte gezeigt werden, dass die orale Bioverfügbarkeit durch lipidbasierte Formulierungen verbessert werden kann, weshalb dieser indirekte Aufnahmeweg, die lymphatische Absorption, bei der Formulierungsentwicklung stärker berücksichtigt werden sollte. Die zusätzliche Methodenetablierung der FACS-Analyse von Lymphflüssigkeit ermöglicht ein besseres Verständnis immunologischer Prozesse nach subkutaner Applikation und kann eingesetzt werden, um die Dynamik der Wechselwirkung großer Moleküle mit dem Immunsystem zu untersuchen.

X. APPENDICES

1 Score sheet

Anhang 4: Scoresheet Nager

Das Scoresheet wird an jedem Tag einer Studie, an dem Symptome beobachtet werden (ausgenommen am Tag der Sektion) ausgefüllt. Dazu wird den Symptomen, die ein Studientier zeigt, anhand der ersten Tabelle ein Punktewert zugeordnet und in der zweiten Tabelle eingetragen. Die Punkte werden dann addiert; Maßnahmen werden in Abhängigkeit vom ermittelten Gesamtscore ergriffen.

	Keine	**Gering (1)**	**Mäßig (2)**	**Schwerwiegend (3)**
Körpergewicht	Gewichtsreduktion </=5%	Gewichtsreduktion </=10%	10 - 20 % Gewichts-reduktion	Gewichts-reduktion >20%
Allgemeinzustand	Aufmerksam, aktiv, offene Augen, rosige Schleimhäute	leicht verminderte Aktivität	Mäßig verminderte Aktivität, teilweiser Lidschluss, Dehydrierung (Hautfalte verstreicht nicht sofort, aber innerhalb von 15 Sekunden), Isolation von der Gruppe	Stark verminderte Aktivität, vollständiger Lidschluss Starke Dehydrierung (Hautfalte nach 15 Sekunden nicht verstrichen), Apathie
Haarkleid / Fell	Normal, saubere Körperöffnungen	Verminderte Fellpflege gesträubtes Fell, ggr. ungepflegtes Fell	Ungepflegtes Haarkleid, Sekretion aus den Augen und Nase, Fell/Augen mittelgr. verschmutzt,	Verklebte oder feuchte Körperöffnungen, Brillenbildung Fell/Augen Hochgr. Verschmutzt, Piloerektion
Hautläsion, Wunde, Trauma	keine	kleinere Wunde	größere Wunden, verschorft	Größere Wunden nässend,
Neurologische Symptome	keine	Tremor, Ataxie (schwankender Gang)	Krämpfe, Einmaliges Auftreten (von kurzer Dauer), Ataxie (schwankender Gang), lang anhaltender Tremor	Krämpfe, mehrmals auftretend (oder länger anhaltend)
Atmung	normal	Leicht beschleunigt	Beschleunigt und verstärkt (abdominal) Atemgeräusch	Beschleunigt, erschwert, persistierendes Atemgeräusch
Schmerzzeichen	keine	-Tippelnder Gang,	Aufgezogener Leib, zusammengekrümmte Körperhaltung,	Automutilation, Aggressivität

			Berührungsempfindlichkeit, z.B. Lautäußerung beim Ergreifen	

Punktestand	
0	Keine Maßnahmen erforderlich
Kategorie gering (1)	Verantwortliche Person informieren. Überwachungsfrequenz alle 12 h. Es sind ggf. Interventionen notwendig (z.B. Analgesie, s.c. Substitution von Flüssigkeit, Boost-Futter). Bei 3 oder mehr Symptomen der Kategorie gering ist von einer mittleren Belastung auszugehen, Anweisungen zu Kategorie mäßig sind auszuführen.
Kategorie mäßig (2)	Das Tier ist der verantwortlichen Person und dem Tierarzt vorzustellen, es sind ggf. Interventionen notwendig (z.B. Analgesie, s.c. Substitution von Flüssigkeit, Boost-Futter). Überwachungsfrequenz alle 8 h. Bei 3 oder mehr Symptomen der Kategorie mäßig ist von einer schwerwiegenden Belastung auszugehen, Anweisungen zu Kategorie schwerwiegend sind auszuführen.
Kategorie schwerwiegend (3)	Versuchsabbruch durch sofortige Tötung

Tiernummer	Körpergewicht	Allgemein-zustand	Haarkleid / Fell	Hautläsionen, Wunden, Trauma	Neurologische Symptome	Atmung	Schmerzzeichen	Summe

Vom Laboranten auszufüllen:

Tierarzt informiert für folgende Tiernummer(n): _____

Datum/ Unterschrift: _____

Vom Tierarzt auszufüllen:

Tierärztliche Kontrolle durchgeführt (Datum, Unterschrift): _____

Behandlung:

- ☐ Nein
- ☐ Ja (Maßnahmen beschreiben): _____
- ☐ Euthanasie

2 Surgery protocol

Datum:				**Zeitpunkt Narkoseeinleitung:**	
Tier:		**Batch ID:**		**Zeitpunkt 1.Metamizolgabe** (100 mg/kg; s.c.):	
Gewicht:				**Zeitpunkt 2.Metamizolgabe** (100 mg/kg; s.c.):	
Zeitpunkt Ölapplikation (0.1 mL):					
Zeitpunkt Itraconazolapplikation:		**Menge:**			
Zeitpunkte Lymphkollektion:	**Uhrzeit:**	**Pulse:**	**Temperature:**	**Respiration rate:**	**Blood pressure:**
baseline					
0 - 15 min					
15 min - 30 min					
30 min -1 hr					
1 hr - 2 hrs					
2 hrs - 3 hrs					
3 hrs - 4 hrs					
4 hrs - 5 hrs					
5 hrs - 6 hrs					
Blutentnahme Necropsy:					

3 Storage stability reports

3.1 Compound 2

General Details	
Compound 2	Bcl-2 Inhibitor
Conc. [mg/ml]	6
Vehicle 1 (non-lipid)	10 % DMSO: 90 % PEG400 (non-lipid)
Vehicle 2 (lipid)	10 % PEG400: 10 % Cremophor EL: 80 % Oleic acid (lipid)
Storage Conditions	
Temperature [°C]	4 °C, RT
Duration	7 days
Light protection (Yes/No)	yes
Comments	n/a

Table 24: *General details and storing conditions of compound 2.*

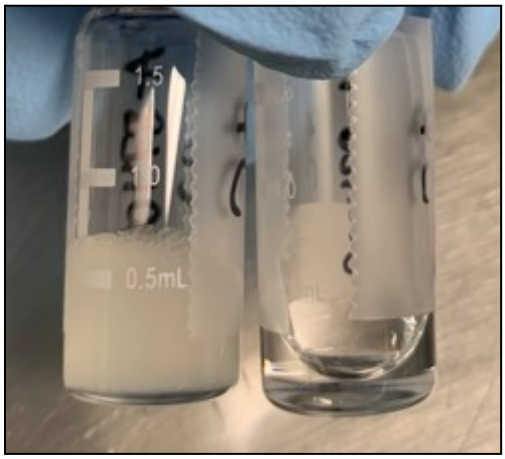
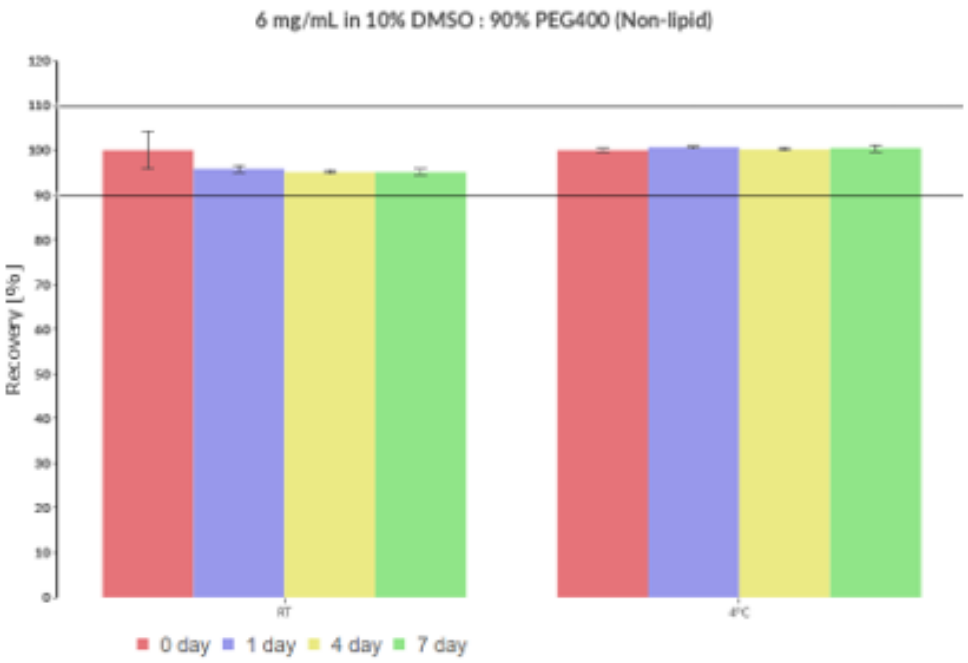


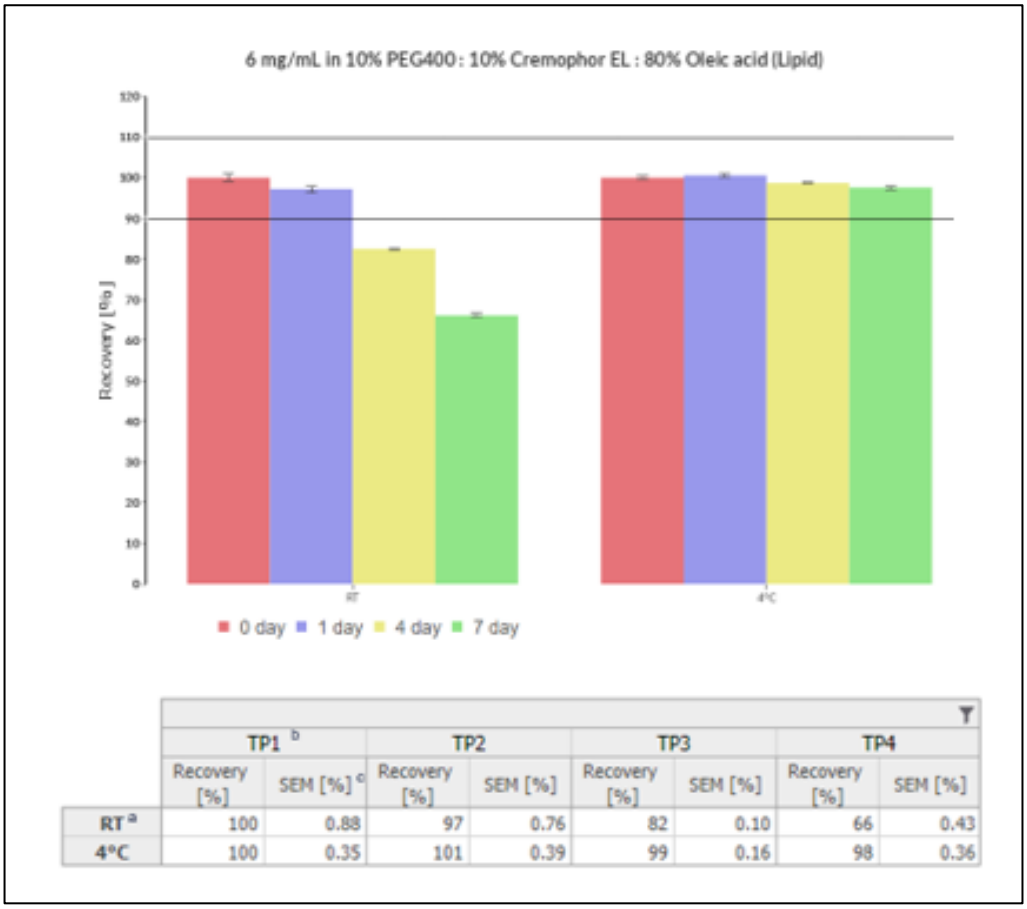
Figure 55: *The compound solution needs to warm up to room temperature (approx. 15 minutes) right before administration, since the formulation in vehicle 2 gets turbid when stored at 4 °C (right bottle).*



	TP1 [b]		TP2		TP3		TP4	
	Recovery [%]	SEM [%] [c]	Recovery [%]	SEM [%]	Recovery [%]	SEM [%]	Recovery [%]	SEM [%]
RT [a]	100	4.16	96	0.75	95	0.39	95	0.62
4°C	100	0.30	101	0.20	100	0.38	101	0.78

Figure 56: *Stability report of the non-lipid based formulation shows in a concentration of 6 mg/ml sufficient stability after 7 days when stored at 4 °C, light protected.*

a. RT: Room temperature
b. TP: Test point
c. SEM: Scanning Electron Microscopy



	TP1 [b]		TP2		TP3		TP4	
	Recovery [%]	SEM [%] [c]	Recovery [%]	SEM [%]	Recovery [%]	SEM [%]	Recovery [%]	SEM [%]
RT [a]	100	0.88	97	0.76	82	0.10	66	0.43
4°C	100	0.35	101	0.39	99	0.16	98	0.36

***Figure 57**: Stability report of the lipid based formulation shows in a concentration of 6 mg/ml sufficient stability after 7 days when stored at 4 °C, light protected.*

a. RT: Room temperature
b. TP: Test point
c. SEM: Scanning Electron Microscopy

3.2 Compound 3

General Details	
Compound 3	Itraconazole
Project Name	n/a
Conc. [mg/ml]	1.5
Vehicle 1	10 % DMSO: 90 % PEG400 (non-lipid)
Vehicle 2	10 % PEG400: 10 % Cremophor EL: 80 % Oleic acid (lipid)
Storage Conditions	
Temperature [°C]	RT and 4 °C
Duration	14 days
Light protection (Yes/No)	yes

***Table 25:** General details and storage conditions of compound 3.*

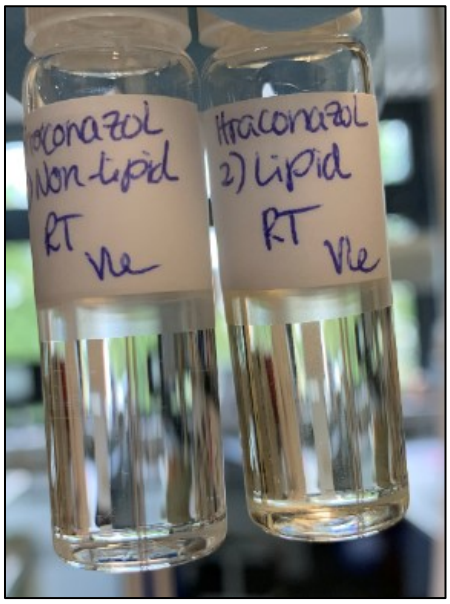


Figure 58: *Non-lipid (right bottle) and lipid based (left bottle) formulation on day 0.*

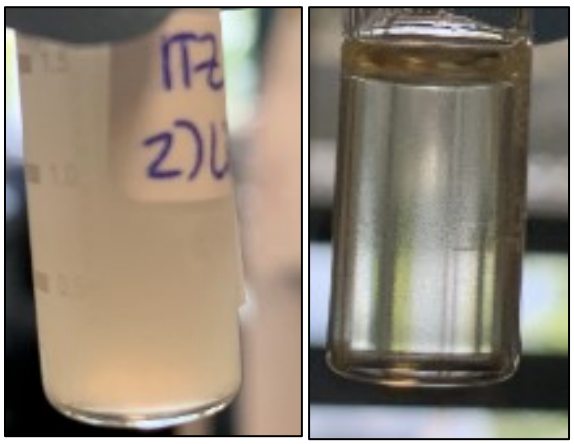
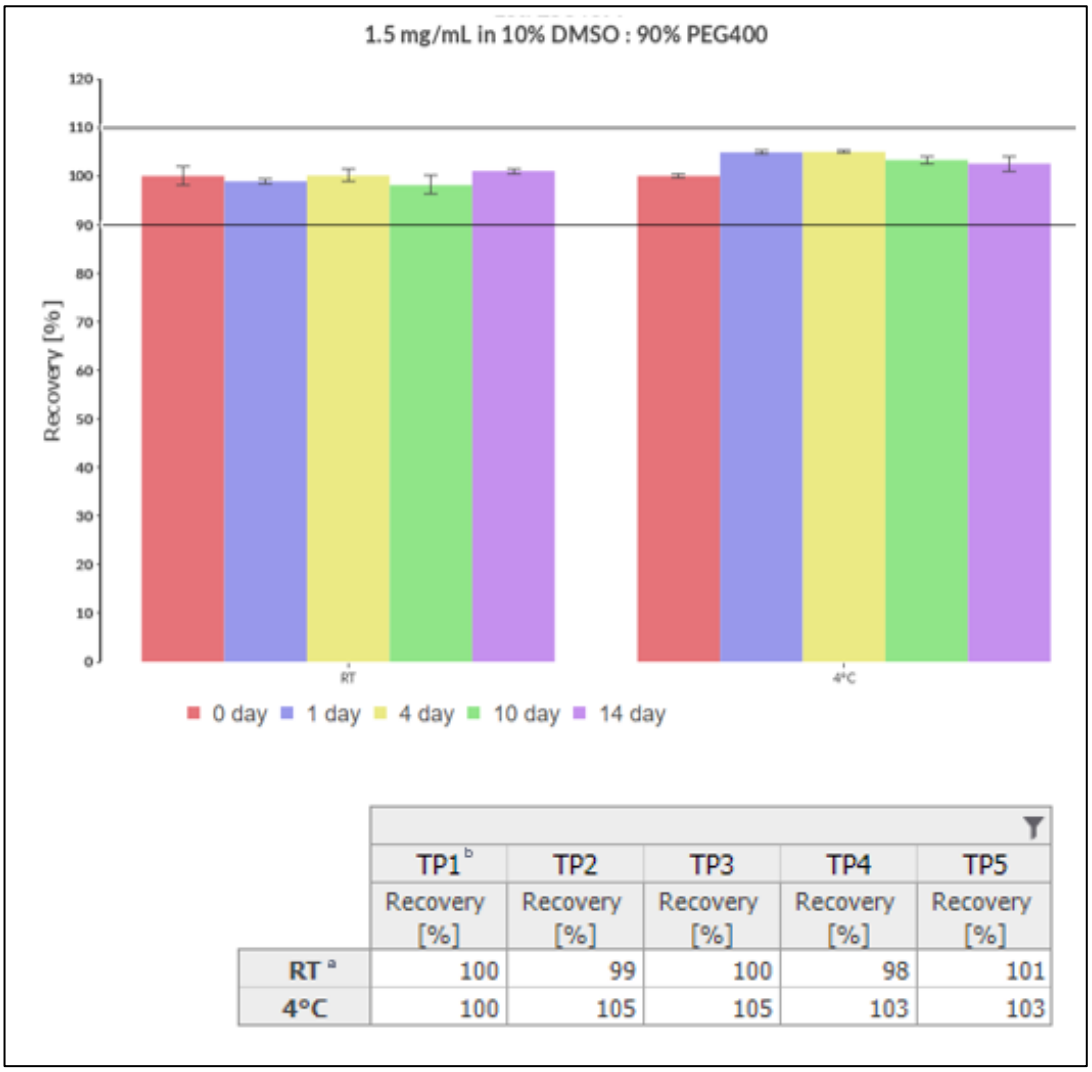


Figure 59 *(left)**:** Lipid formulation before and* ***Figure 60*** *(right): after ultrasonication stored at 4 °C after 14 days.*

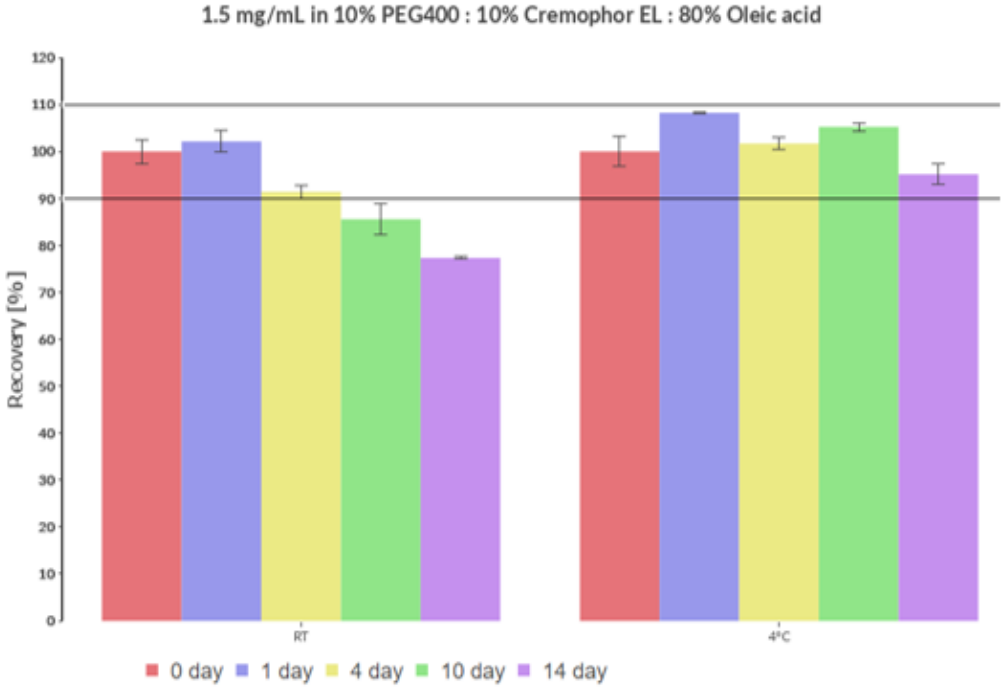
Figure 61: *Discolored lipid formulation stored at room temperature after 10 days.*



	TP1 [b]	TP2	TP3	TP4	TP5
	Recovery [%]	Recovery [%]	Recovery [%]	Recovery [%]	Recovery [%]
RT [a]	100	99	100	98	101
4°C	100	105	105	103	103

Figure 62: *Non-lipid formulation, the compound shows sufficient physical stability after 14 days stored at room temperature and 4 °C.*

a. RT: Room temperature
b. TP: Test point



	TP1[b]	TP2	TP3	TP4	TP5
	Recovery [%]	Recovery [%]	Recovery [%]	Recovery [%]	Recovery [%]
RT[a]	100	102	91	86	77
4°C	100	108	102	105	95

***Figure 63**: Lipid formulation, the compound solution shows sufficient physical stability after 24 hours stored at room temperature. It shows sufficient chemical stability after 4 days stored at RT and after 14 days stored at 4 °C, light protected.*

a. RT: Room temperature
b. TP: Test point

4 Immunophenotyping protocol

Immunophenotyping 1 / 2

TAXX-XXX_Rat-Preparation

- 1. Isotype Control-Cocktail ansetzen
  je Control-Tube 50 µl Stain Buffer (BSA) + 32 µl Isotyp-Ansatz (siehe Tabelle)

1. Antikörper-Cocktail ansetzen
   je AK-Tube 50 µl Stain Buffer (BSA) + 32 µl AK-Ansatz (siehe Tabelle)

1. FMO - Controls ansetzen
   je FMO 50 µl Stain Buffer (BSA) + AK-Ansatz (siehe Tabelle)

- 2. mischen

labeled sample tube oder 96er Deep - Platte

- 3. in jedes Tube 100 µl Blut geben
- 4. in jedes Tube 52 µl FC Block geben
  (50 µl Stain Buffer + 2 µl CD32)
  31 Ansätze: 1550 µl Stain Buffer (BSA) + 62 µl CD32, 550271

5. mischen

- 6. 5 min bei Raumtemperatur inkubieren
- 7. in jedes Tube 82 µl Isotype oder AK Cocktail geben, bzw. FMO Kontrollen (s. 1.)
- 8. mischen
- 9. 30 min inkubieren bei Raumtemperatur und dunkel
- 10. in jedes Tube 2 ml PBS geben
- 11. mischen
- 12. 5 min bei 300 - 400g zentrifugieren
- 13. Überstand abkippen
- 14. in jedes Tube 2 ml PBS geben
- 15. mischen
- 16. 5 min bei 300 - 400g zentrifugieren

- 17. Überstand abkippen
- 18. in jedes Tube 200 µl Fixable viable dye 780 - Ansatz geben
  (FVS 780 dye powder -80° + 180 µl DMSO Dimethyl Sulfoxide
  = Aliquots bei -20°;
  10 µl FVS 780 aus Aliquots + 10000 µl PBS = FVS 780 - Ansatz)
- 19. 20 min. inkubieren bei Raumtemperatur und dunkel
- 20. in jedes Tube 2 ml PBS geben
- 21. 5 min bei 300 - 400g zentrifugieren
- 22. abkippen
- 23. in jedes Tube 2 ml Lyse/Fix-Ansatz geben
  (für 100 ml Ansatz: 20 ml Lyse/Fix Buffer + 80 ml H2O)
- 24. gut mischen (zur vollständigen RBC-Lyse)
- 25. 10 - 15 min bei 37° C im Dunkeln inkubieren
- 26. 5 min bei 300 - 400g zentrifugieren
- 27. Überstand abkippen
- 28. 2 ml kalter Stain Buffer (BSA) zugeben
- 29. mischen
- 30. 5 min bei 300 - 400g zentrifugieren
- 31. Überstand abkippen
- 32. 500 µl Stain Buffer (BSA) zugeben
- 33. mischen
- 34. bis zur Analyse vor Licht schützen;
  zur Lagerung länger als 6 Std. in Kühlschrank
- 35. vor dem Messen mischen
- 36. 10 000 Zellen (Lymphocyten) messen

5 Tissue obstructing catheter

Figure 64 *(left): Obstruction within the catheter, piece of tissue interrupting lymph flow (black arrow) and* ***Figure 65*** *(right)****:*** *Piece of tissue from the catheter on a swab (red arrow).*

6 Supplementary data

6.1 Cell size

	Relative size [Median FCS-A], baseline blood	Relative size [Median FCS-A], baseline lymph	Percentage difference (blood vs lymph)
Animal 1	105152	97792	-7.00
Animal 2	104576	84224	-19.46
Animal 4	102528	71168	-30.59
Animal 5	100288	88320	-11.93
Animal 6	101120	89984	-11.01

Table 26: *Relative cell size of lymphocytes in baseline lymph and relative cell size of lymphocytes in baseline blood of group 2.*

Table Analyzed	**Col: Paired t-test**
Column B	lymph
vs.	vs,
Column A	blood
Paired t-test	
P value	0.0285
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 3.976, df = 3
Number of pairs	4
How big is the difference?	
Mean of differences (B - A)	-18704
SD of differences	9408
SEM of differences	4704
95 % confidence interval	-33674 to -3734
R squared (partial eta squared)	0.8405
How effective was the pairing?	
Correlation coefficient (r)	-0.3872
P value (one tailed)	0.3064
P value summary	ns
Was the pairing significantly effective?	No

Table 27: *Paired t-test comparing relative cell size of lymphocytes in baseline lymph samples to baseline blood samples in group 2 (n = 5).*

6.2 Comparison of cell populations in baseline lymph and baseline blood of group 2

Due to unexplained cell degradation in 30 minute samples from animal 9 and 15 minutes sample of animals 11 of group 1, the values of these two samples were not included in the statistical analysis.

Lymphocytes

Table Analyzed	**Col: Paired t-test**
Column B	lymph
vs.	vs,
Column A	blood
Paired t-test	
P value	< 0.0001
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 25.42, df = 4
Number of pairs	5
How big is the difference?	
Mean of differences (B - A)	48.58
SD of differences	4.273
SEM of differences	1.911
95 % confidence interval	43.27 to 53.89
R squared (partial eta squared)	0.9938
How effective was the pairing?	
Correlation coefficient (r)	0.5051
P value (one tailed)	0.1927
P value summary	ns
Was the pairing significantly effective?	No

***Table 28**: Paired t-test comparing amount of lymphocytes in baseline lymph samples to baseline blood samples in group 2 (n = 5).*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 7	49.7	54.8	24.2	23.8	17.2	21.3
Animal 9	57.4	43.2		31.7	56.6	28.5
Animal 11	53.9		33.6	19.7	12.4	15.6
Animal 12	41	32.4	36	28.3	21.1	24.5
Number of values	4	3	3	4	4	4
Minimum	41	32.4	24.2	19.7	12.4	15.6
25 % Percentile	43.18	32.4	24.2	20.73	13.6	17.03
Median	51.8	43.2	33.6	26.05	19.15	22.9
75 % Percentile	56.53	54.8	36	30.85	47.73	27.5
Maximum	57.4	54.8	36	31.7	56.6	28.5
Range	16.4	22.4	11.8	12	44.2	12.9
Mean	50.5	43.47	31.27	25.88	26.83	22.48
Std. Deviation	7.072	11.2	6.236	5.236	20.17	5.448
Std. Error of Mean	3.536	6.468	3.601	2.618	10.08	2.724
Lower 95 % CI of mean	39.25	15.64	15.77	17.54	-5.264	13.81
Upper 95 % CI of mean	61.75	71.29	46.76	34.21	58.91	31.14

Table 29: *Lymphocytes in blood samples of group 1, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 1	96.7	96.4	93.4	94.5	93.8	92.5
Animal 2	96	93.2	93.2	94.5	94.3	92.6
Animal 4	94.6	95.7	95	93.4	94.1	93.7
Animal 5	97.1	94.9	92.9	96.2	95.8	93.8
Animal 6	97	96.2	96.1	93.4	97.4	94.9
Number of values	5	5	5	5	5	5
Minimum	94,60	93,20	92,90	93,40	93,80	92,50
25 % Percentile	95,30	94,05	93,05	93,40	93,95	92,55
Median	96,70	95,70	93,40	94,50	94,30	93,70
75 % Percentile	97,05	96,30	95,55	95,35	96,60	94,35
Maximum	97,10	96,40	96,10	96,20	97,40	94,90
Range	2,500	3,200	3,200	2,800	3,600	2,400
Mean	96,28	95,28	94,12	94,40	95,08	93,50
Std. Deviation	1,033	1,299	1,374	1,147	1,509	0,9874
Std. Error of Mean	0,4620	0,5809	0,6143	0,5128	0,6748	0,4416
Lower 95 % CI of mean	95,00	93,67	92,41	92,98	93,21	92,27
Upper 95 % CI of mean	97,56	96,89	95,83	95,82	96,95	94,73

Table 30: *Lymphocytes in lymph fluid of group, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

Granulocytes

Table Analyzed	**Col: Paired t-test**
Column B	lymph
vs.	vs,
Column A	blood
Paired t-test	
P value	0.0019
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 7.258, df = 4
Number of pairs	5
How big is the difference?	
Mean of differences (B - A)	-8.034
SD of differences	2.475
SEM of differences	1.107
95 % confidence interval	-11.11 to -4.960
R squared (partial eta squared)	0.9294
How effective was the pairing?	
Correlation coefficient (r)	0.6064
P value (one tailed)	0.1391
P value summary	ns
Was the pairing significantly effective?	No

Table 31: *Paired t-test comparing amount of granulocytes in baseline lymph samples to baseline blood samples in group 2 (n = 5).*

	baseline	**15 minutes**	**30 minutes**	**1 hour**	**2 hours**	**3 hours**
Animal 7	7.93	3.68	11.5	15.7	18	28.2
Animal 9	1.67	11.9		7.45	2.97	11.3
Animal 11	7.16		12.1	14.2	16.4	22.2
Animal 12	14.3	10.8	9.77	13.6	17.7	21.1
Number of values	4	3	3	4	4	4
Minimum	1.67	3.68	9.77	7.45	2.97	11.3
25 % Percentile	3.043	3.68	9.77	8.988	6.328	13.75
Median	7.545	10.8	11.5	13.9	17.05	21.65
75 % Percentile	12.71	11.9	12.1	15.33	17.93	26.7
Maximum	14.3	11.9	12.1	15.7	18	28.2
Range	12.63	8.22	2.33	8.25	15.03	16.9
Mean	7.765	8.793	11.12	12.74	13.77	20.7
Std. Deviation	5.172	4.462	1.21	3.634	7.232	7
Std. Error of Mean	2.586	2.576	0.6985	1.817	3.616	3.5
Lower 95 % CI of mean	-0.4648	-2.292	8.118	6.955	2.26	9.561
Upper 95 % CI of mean	15.99	19.88	14.13	18.52	25.27	31.84

Table 32: *Granulocytes in blood samples of group 1, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 1	0.01	0.021	0.011	0.051	0.01	0.052
Animal 2	0.061	0.051	0.1	0.12	0.11	0.15
Animal 4	0.11	0	0.022	0.034	0.022	0.25
Animal 5	0.01	0.021	0.011	0.051	0.01	0.052
Animal 6	0.061	0.051	0.1	0.12	0.11	0.15
Number of values	5	5	5	5	5	5
Minimum	0.01	0	0.011	0.034	0.01	0.052
25 % Percentile	0.01	0.0105	0.011	0.0425	0.01	0.052
Median	0.061	0.021	0.022	0.051	0.022	0.15
75 % Percentile	0.0855	0.051	0.1	0.12	0.11	0.2
Maximum	0.11	0.051	0.1	0.12	0.11	0.25
Range	0.1	0.051	0.089	0.086	0.1	0.198
Mean	0.0504	0.0288	0.0488	0.0752	0.0524	0.1308
Std. Deviation	0.04196	0.022	0.04695	0.04148	0.05281	0.08271
Std. Error of Mean	0.01876	0.009841	0.021	0.01855	0.02362	0.03699
Lower 95 % CI of mean	-0.001695	0.001478	-0.009501	0.02369	-0.01317	0.0281
Upper 95 % CI of mean	0.1025	0.05612	0.1071	0.1267	0.118	0.2335

Table 33: *Granulocytes in lymph fluid of group 2, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

Monocytes

Table Analyzed	**Col: Paired t-test**
Column B	lymph
vs.	vs,
Column A	blood
Paired t-test	
P value	0.0003
P value summary	***
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 11.54, df = 4
Number of pairs	5
How big is the difference?	
Mean of differences (B - A)	-2.646
SD of differences	0.5126
SEM of differences	0.2292
95 % confidence interval	-3.282 to -2.010
R squared (partial eta squared)	0.9709
How effective was the pairing?	
Correlation coefficient (r)	0.005076
P value (one tailed)	0.4968
P value summary	ns
Was the pairing significantly effective?	No

Table 34: *Paired t-test comparing amount of monocytes in baseline lymph samples to baseline blood samples in group 2 (n = 5).*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 7	3.36	2.1	3.32	3.39	4.1	5.61
Animal 9	0.85	3.18		2.18	0.83	3.35
Animal 11	3.75		3.01	2.02	4.01	4.4
Animal 12	3.83	4.17	3.77	3.71	6.31	4.28
Number of values	4	3	3	4	4	4
Minimum	0.85	2.1	3.01	2.02	0.83	3.35
25 % Percentile	1.478	2.1	3.01	2.06	1.625	3.583
Median	3.555	3.18	3.32	2.785	4.055	4.34
75 % Percentile	3.81	4.17	3.77	3.63	5.758	5.308
Maximum	3.83	4.17	3.77	3.71	6.31	5.61
Range	2.98	2.07	0.76	1.69	5.48	2.26
Mean	2.948	3.15	3.367	2.825	3.813	4.41
Std. Deviation	1.413	1.035	0.3821	0.8498	2.255	0.9275
Std. Error of Mean	0.7067	0.5977	0.2206	0.4249	1.127	0.4637
Lower 95 % CI of mean	0.6986	0.5781	2.417	1.473	0.2244	2.934
Upper 95 % CI of mean	5.196	5.722	4.316	4.177	7.401	5.886

***Table 35**: Monocytes in blood samples of group 1, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 1	0.14	0.052	0.32	0.078	0.17	0.34
Animal 2	0.29	0.73	0.96	0.42	0.19	0.81
Animal 4	0.13	0.17	0.11	0.16	0.11	0.33
Animal 5	0.12	0.6	0.94	0.25	0.38	0.54
Animal 6	0.18	0.25	0.18	0.27	0.16	0.44
Number of values	5	5	5	5	5	5
Minimum	0.12	0.052	0.11	0.078	0.11	0.33
25 % Percentile	0.125	0.111	0.145	0.119	0.135	0.335
Median	0.14	0.25	0.32	0.25	0.17	0.44
75 % Percentile	0.235	0.665	0.95	0.345	0.285	0.675
Maximum	0.29	0.73	0.96	0.42	0.38	0.81
Range	0.17	0.678	0.85	0.342	0.27	0.48
Mean	0.172	0.3604	0.502	0.2356	0.202	0.492
Std. Deviation	0.06979	0.2905	0.416	0.1284	0.1038	0.1972
Std. Error of Mean	0.03121	0.1299	0.186	0.05742	0.04641	0.08817
Lower 95 % CI of mean	0.08535	-0.0003046	-0.01448	0.07617	0.07314	0.2472
Upper 95 % CI of mean	0.2587	0.7211	1.018	0.395	0.3309	0.7368

***Table 36:** Monocytes in lymph fluid of group 2, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

T-cells

Table Analyzed	Col: Paired t-test
Column B	lymph
vs.	vs,
Column A	blood
Paired t-test	
P value	0,0002
P value summary	***
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 12.74, df = 4
Number of pairs	5
How big is the difference?	
Mean of differences (B - A)	41.32
SD of differences	7.252
SEM of differences	3.243
95 % confidence interval	32.32 to 50.32
R squared (partial eta squared)	0.9760
How effective was the pairing?	
Correlation coefficient (r)	0.4475
P value (one tailed)	0.2249
P value summary	ns
Was the pairing significantly effective?	No

Table 37: *Paired t-test comparing amount of T-cells in baseline lymph samples to baseline blood samples in group 2 (n = 5).*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 7	53.5	32.1	22.9	19.6	17	11.2
Animal 9	44	49.5		44.5	42.5	33.1
Animal 11	52.8		20.2	18.2	13.8	11.2
Animal 12	44.8	26.2	16.4	14.5	7.76	8.03
Number of values	4	3	3	4	4	4
Minimum	44	26.2	16.4	14.5	7.76	8.03
25 % Percentile	44.2	26.2	16.4	15.43	9.27	8.823
Median	48.8	32.1	20.2	18.9	15.4	11.2
75 % Percentile	53.33	49.5	22.9	38.28	36.13	27.63
Maximum	53.5	49.5	22.9	44.5	42.5	33.1
Range	9.5	23.3	6.5	30	34.74	25.07
Mean	48.78	35.93	19.83	24.2	20.27	15.88
Std. Deviation	5.07	12.11	3.265	13.7	15.31	11.58
Std. Error of Mean	2.535	6.994	1.885	6.852	7.655	5.788
Lower 95 % CI of mean	40.71	5.841	11.72	2.395	-4.097	-2.536
Upper 95 % CI of mean	56.84	66.03	27.95	46	44.63	34.3

Table 38: *T-cells in blood samples of group 1, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 1	89	86.2	87.6	86.4	89.3	88.7
Animal 2	89.5	91	91.8	92.4	95.1	95.4
Animal 4	84.4	83.5	86.6	87.7	88.9	89.8
Animal 5	91.5	91.7	94.5	92.1	93	94.6
Animal 6	95.7	92.8	93	93.8	94.5	92.4
Number of values	5	5	5	5	5	5
Minimum	84.4	83.5	86.6	86.4	88.9	88.7
25 % Percentile	86.7	84.85	87.1	87.05	89.1	89.25
Median	89.5	91	91.8	92.1	93	92.4
75 % Percentile	93.6	92.25	93.75	93.1	94.8	95
Maximum	95.7	92.8	94.5	93.8	95.1	95.4
Range	11.3	9.3	7.9	7.4	6.2	6.7
Mean	90.02	89.04	90.7	90.48	92.16	92.18
Std. Deviation	4.103	3.994	3.441	3.229	2.9	2.918
Std. Error of Mean	1.835	1.786	1.539	1.444	1.297	1.305
Lower 95 % CI of mean	84.93	84.08	86.43	86.47	88.56	88.56
Upper 95 % CI of mean	95.11	94	94.97	94.49	95.76	95.8

Table 39: *T-cells in lymph fluid of group 2, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

T-helper cells

Table Analyzed	**Col: Paired t-test**
Column B	lymph
vs.	vs,
Column A	blood
Paired t-test	
P value	0.0322
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 3.221, df = 4
Number of pairs	5
How big is the difference?	
Mean of differences (B - A)	6.720
SD of differences	4.665
SEM of differences	2.086
95 % confidence interval	0.9277 to 12.51
R squared (partial eta squared)	0.7217
How effective was the pairing?	
Correlation coefficient (r)	-0.1896
P value (one tailed)	0.3800
P value summary	ns
Was the pairing significantly effective?	No

Table 40: *Paired t-test comparing amount of T-helper cells in baseline lymph samples to baseline blood samples in group 2 (n = 5).*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 7	70.3	79.2	78.4	68.1	74.2	69.7
Animal 9	72.3	66.6		69.8	74.7	74.2
Animal 11	67.8		79	75.2	70.9	69.2
Animal 12	70.3	76.7	82.1	75.1	72	72
Number of values	4	3	3	4	4	4
Minimum	67.8	66.6	78.4	68.1	70.9	69.2
25 % Percentile	68.43	66.6	78.4	68.53	71.18	69.33
Median	70.3	76.7	79	72.45	73.1	70.85
75 % Percentile	71.8	79.2	82.1	75.18	74.58	73.65
Maximum	72.3	79.2	82.1	75.2	74.7	74.2
Range	4.5	12.6	3.7	7.1	3.8	5
Mean	70.18	74.17	79.83	72.05	72.95	71.28
Std. Deviation	1.843	6.671	1.986	3.646	1.801	2.3
Std. Error of Mean	0.9214	3.852	1.146	1.823	0.9005	1.15
Lower 95 % CI of mean	67.24	57.59	74.9	66.25	70.08	67.62
Upper 95 % CI of mean	73.11	90.74	84.77	77.85	75.82	74.93

Table 41: *T-helper cells in blood samples of group 1, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 1	75.3	76.2	74.1	78.6	77.1	76
Animal 2	72.7	69.7	67.9	74.6	77.4	76.8
Animal 4	73.9	71	72.4	76.6	80.3	77.3
Animal 5	80.2	77.8	79.4	82.7	82	84.3
Animal 6	78.1	76	75.9	73.5	77.8	77.1
Number of values	5	5	5	5	5	5
Minimum	72.7	69.7	67.9	73.5	77.1	76
25 % Percentile	73.3	70.35	70.15	74.05	77.25	76.4
Median	75.3	76	74.1	76.6	77.8	77.1
75 % Percentile	79.15	77	77.65	80.65	81.15	80.8
Maximum	80.2	77.8	79.4	82.7	82	84.3
Range	7.5	8.1	11.5	9.2	4.9	8.3
Mean	76.04	74.14	73.94	77.2	78.92	78.3
Std. Deviation	3.075	3.559	4.258	3.641	2.137	3.39
Std. Error of Mean	1.375	1.592	1.904	1.628	0.9557	1.516
Lower 95 % CI of mean	72.22	69.72	68.65	72.68	76.27	74.09
Upper 95 % CI of mean	79.86	78.56	79.23	81.72	81.57	82.51

Table 42: *T-helper cells in lymph fluid of group 2, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

B-cells

Table Analyzed	Col: Paired t-test
Column B	lymph
vs.	vs,
Column A	blood
Paired t-test	
P value	0.0007
P value summary	***
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 9.345, df = 4
Number of pairs	5
How big is the difference?	
Mean of differences (B - A)	-27.75
SD of differences	6.639
SEM of differences	2.969
95 % confidence interval	-35.99 to -19.50
R squared (partial eta squared)	0.9562
How effective was the pairing?	
Correlation coefficient (r)	0.8129
P value (one tailed)	0.0472
P value summary	*
Was the pairing significantly effective?	Yes

***Table 43**: Paired t-test comparing amount of B-cells cells in baseline lymph samples to baseline blood samples in group 2 (n = 5).*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 7	34	42.6	46.3	54.9	65.5	76.3
Animal 9	44.4	33.8		38.9	45.3	53.9
Animal 11	34.9		58.6	57	67.9	73.1
Animal 12	42.3	56.8	65.7	64.4	76.9	82.9
Number of values	4	3	3	4	4	4
Minimum	34	33.8	46.3	38.9	45.3	53.9
25 % Percentile	34.23	33.8	46.3	42.9	50.35	58.7
Median	38.6	42.6	58.6	55.95	66.7	74.7
75 % Percentile	43.88	56.8	65.7	62.55	74.65	81.25
Maximum	44.4	56.8	65.7	64.4	76.9	82.9
Range	10.4	23	19.4	25.5	31.6	29
Mean	38.9	44.4	56.87	53.8	63.9	71.55
Std. Deviation	5.222	11.61	9.815	10.74	13.34	12.45
Std. Error of Mean	2.611	6.7	5.667	5.368	6.668	6.227
Lower 95 % CI of mean	30.59	15.57	32.48	36.72	42.68	51.73
Upper 95 % CI of mean	47.21	73.23	81.25	70.88	85.12	91.37

***Table 44:** B-cells in blood samples of group 1, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 1	9.58	12.5	10.9	12	9.09	9.83
Animal 2	8.35	6.52	5.02	5.92	3.29	2.81
Animal 4	12.9	13	10.5	10.2	8.97	7.49
Animal 5	6.98	7.42	4.63	6.97	6.14	4.25
Animal 6	3.16	5.69	5.79	4.85	4.55	5.91
Number of values	5	5	5	5	5	5
Minimum	3.16	5.69	4.63	4.85	3.29	2.81
25 % Percentile	5.07	6.105	4.825	5.385	3.92	3.53
Median	8.35	7.42	5.79	6.97	6.14	5.91
75 % Percentile	11.24	12.75	10.7	11.1	9.03	8.66
Maximum	12.9	13	10.9	12	9.09	9.83
Range	9.74	7.31	6.27	7.15	5.8	7.02
Mean	8.194	9.026	7.368	7.988	6.408	6.058
Std. Deviation	3.567	3.459	3.073	3.006	2.598	2.744
Std. Error of Mean	1.595	1.547	1.374	1.344	1.162	1.227
Lower 95 % CI of mean	3.764	4.731	3.552	4.255	3.182	2.651
Upper 95 % CI of mean	12.62	13.32	11.18	11.72	9.634	9.465

Table 45: *B-cells in lymph fluid of group 2, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

NK-cells

Table Analyzed	**Col: Paired t-test**
Column B	lymph
vs.	vs,
Column A	blood
Paired t-test	
P value	0.0012
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 8.252, df = 4
Number of pairs	5
How big is the difference?	
Mean of differences (B - A)	-3.084
SD of differences	0.8358
SEM of differences	0,3738
95 % confidence interval	-4.122 to -2.047
R squared (partial eta squared)	0.9445
How effective was the pairing?	
Correlation coefficient (r)	-0.1494
P value (one tailed)	0.4052
P value summary	ns
Was the pairing significantly effective?	No

Table 46: *Paired t-test comparing amount of NK-cells cells in baseline lymph samples to baseline blood samples in group 2 (n = 5).*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 7	3.27	8.52	10.1	10.6	8.04	5.33
Animal 9	2.1	2.78		2.98	2.73	2.83
Animal 11	3.64		5.57	7.95	6.43	5.86
Animal 12	3.16	4.91	5.85	7.55	6.37	3.18
Number of values	4	3	3	4	4	4
Minimum	2.1	2.78	5.57	2.98	2.73	2.83
25 % Percentile	2.365	2.78	5.57	4.123	3.64	2.918
Median	3.215	4.91	5.85	7.75	6.4	4.255
75 % Percentile	3.548	8.52	10.1	9.938	7.638	5.728
Maximum	3.64	8.52	10.1	10.6	8.04	5.86
Range	1.54	5.74	4.53	7.62	5.31	3.03
Mean	3.043	5.403	7.173	7.27	5.893	4.3
Std. Deviation	0.661	2.902	2.538	3.164	2.246	1.518
Std. Error of Mean	0.3305	1.675	1.466	1.582	1.123	0.7588
Lower 95 % CI of mean	1.991	-1.805	0.8675	2.235	2.319	1.885
Upper 95 % CI of mean	4.094	12.61	13.48	12.3	9.466	6.715

Table 47: *NK-cells in blood samples of group 1, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 1	0.19	0.065	0.17	0	0.034	0.071
Animal 2	0.2	0.18	0.23	0.11	0.057	0.093
Animal 4	0.035	0.068	0.25	0.062	0.024	0.049
Animal 5	0.042	0.044	0.023	0.022	0.011	0.023
Animal 6	0.011	0.075	0.032	0.057		0.034
Number of values	5	5	5	5	5	5
Minimum	0.011	0.044	0.023	0	0.011	0.023
25 % Percentile	0.023	0.0545	0.0275	0.011	0.011	0.0285
Median	0.042	0.068	0.17	0.057	0.024	0.049
75 % Percentile	0.195	0.1275	0.24	0.086	0.0455	0.082
Maximum	0.2	0.18	0.25	0.11	0.057	0.093
Range	0.189	0.136	0.227	0.11	0.046	0.07
Mean	0.0956	0.0864	0.141	0.0502	0.0274	0.054
Std. Deviation	0.09153	0.05358	0.1078	0.04206	0.01917	0.02827
Std. Error of Mean	0.04093	0.02396	0.04819	0.01881	0.008571	0.01264
Lower 95 % CI of mean	-0.01805	0.01987	0.0072	-0.002027	0.003603	0.0189
Upper 95 % CI of mean	0.2093	0.1529	0.2748	0.1024	0.0512	0.0891

Table 48: *NK-cells in lymph fluid of group 2, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

T-killer cells

Table Analyzed	**Col: Paired t-test**
Column B	lymph
vs.	vs,
Column A	blood
Paired t-test	
P value	0.0279
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 3.375, df = 4
Number of pairs	5
How big is the difference?	
Mean of differences (B - A)	-6.140
SD of differences	4.069
SEM of differences	1.820
95 % confidence interval	-11.19 to -1.088
R squared (partial eta squared)	0.7400
How effective was the pairing?	
Correlation coefficient (r)	-0.08531
P value (one tailed)	0.4458
P value summary	ns
Was the pairing significantly effective?	No

Table 49: *Paired t-test comparing amount of T-killer cells in baseline lymph samples to baseline blood samples in group 2 (n = 5).*

	baseline	**15 minutes**	**30 minutes**	**1 hour**	**2 hours**	**3 hours**
Animal 7	27.4	18.8	19.1	29.5	23.8	27.9
Animal 9	26.4	31.6		28.3	23.9	23.7
Animal 11	29.8		18	22.1	25.4	28.3
Animal 12	26.9	20.8	16	22.4	23.9	24.5
Number of values	4	3	3	4	4	4
Minimum	26.4	18.8	16	22.1	23.8	23.7
25 % Percentile	26.53	18.8	16	22.18	23.83	23.9
Median	27.15	20.8	18	25.35	23.9	26.2
75 % Percentile	29.2	31.6	19.1	29.2	25.03	28.2
Maximum	29.8	31.6	19.1	29.5	25.4	28.3
Range	3.4	12.8	3.1	7.4	1.6	4.6
Mean	27.63	23.73	17.7	25.58	24.25	26.1
Std. Deviation	1.506	6.886	1.572	3.872	0.7681	2.338
Std. Error of Mean	0.7532	3.975	0.9074	1.936	0.3841	1.169
Lower 95 % CI of mean	25.23	6.628	13.8	19.41	23.03	22.38
Upper 95 % CI of mean	30.02	40.84	21.6	31.74	25.47	29.82

Table 50: *T-killer cells in blood samples of group 1, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 1	22.9	22.2	24.1	19.6	21.4	22.5
Animal 2	25.7	28.7	30.8	24	21.3	22.3
Animal 4	23.6	26.6	25.6	21.6	17.8	21.3
Animal 5	17.8	20.2	18.5	15.8	16.4	14.4
Animal 6	20.5	22.6	22.8	25	20.8	21.1
Number of values	5	5	5	5	5	5
Minimum	17.8	20.2	18.5	15.8	16.4	14.4
25 % Percentile	19.15	21.2	20.65	17.7	17.1	17.75
Median	22.9	22.6	24.1	21.6	20.8	21.3
75 % Percentile	24.65	27.65	28.2	24.5	21.35	22.4
Maximum	25.7	28.7	30.8	25	21.4	22.5
Range	7.9	8.5	12.3	9.2	5	8.1
Mean	22.1	24.06	24.36	21.2	19.54	20.32
Std. Deviation	3.037	3.481	4.468	3.68	2.293	3.365
Std. Error of Mean	1.358	1.557	1.998	1.646	1.025	1.505
Lower 95 % CI of mean	18.33	19.74	18.81	16.63	16.69	16.14
Upper 95 % CI of mean	25.87	28.38	29.91	25.77	22.39	24.5

T***able 51:*** *T-killer cells in lymph fluid of group 2, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

NK T-cells

Table Analyzed	**Col: Paired t-test**
Column B	lymph
vs.	vs,
Column A	blood
Paired t-test	
P value	0.3450
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t = 1070, df = 4
Number of pairs	5
How big is the difference?	
Mean of differences (B - A)	1.640
SD of differences	3.428
SEM of differences	1.533
95 % confidence interval	-2.617 to 5.897
R squared (partial eta squared)	0.2224
How effective was the pairing?	
Correlation coefficient (r)	0.4494
P value (one tailed)	0.2238
P value summary	ns
Was the pairing significantly effective?	No

Table 52: *Paired t-test comparing amount of NK T-cells in baseline lymph samples to baseline blood samples in group 2 (n = 5).*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 7	1.64	1.44	1.35	1.62	0.65	0.4
Animal 9	2.6	3.94		3.81	2.79	1.88
Animal 11	1.43		1.77	1.16	0.57	0.45
Animal 12	2.62	1.27	1.01	0.83	0.34	0.25
Number of values	4	3	3	4	4	4
Minimum	1.43	1.27	1.01	0.83	0.34	0.25
25 % Percentile	1.483	1.27	1.01	0.9125	0.3975	0.2875
Median	2.12	1.44	1.35	1.39	0.61	0.425
75 % Percentile	2.615	3.94	1.77	3.263	2.255	1.523
Maximum	2.62	3.94	1.77	3.81	2.79	1.88
Range	1.19	2.67	0.76	2.98	2.45	1.63
Mean	2.073	2.217	1.377	1.855	1.088	0.745
Std. Deviation	0.6266	1.495	0.3807	1.343	1.143	0.7614
Std. Error of Mean	0.3133	0.8631	0.2198	0.6715	0.5713	0.3807
Lower 95 % CI of mean	1.075	-1.497	0.431	-0.282	-0.7306	-0.4666
Upper 95 % CI of mean	3.07	5.93	2.322	3.992	2.906	1.957

Table 53: *NK T-cells in blood samples of group 1, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 1	7.45	4.1	9.67	19	5.79	13.4
Animal 2	9.24	5.66	4.65	7.7	6.08	3.15
Animal 4	3.56	1.76	2.24	3.99	2.41	2.47
Animal 5	0.92	1.03	0.42	0.7	0.92	1.57
Animal 6	1.3	1.74	1.54	2.36	0.89	1.54
Number of values	5	5	5	5	5	5
Minimum	0.92	1.03	0.42	0.7	0.89	1.54
25 % Percentile	1.11	1.385	0.98	1.53	0.905	1.555
Median	3.56	1.76	2.24	3.99	2.41	2.47
75 % Percentile	8.345	4.88	7.16	13.35	5.935	8.275
Maximum	9.24	5.66	9.67	19	6.08	13.4
Range	8.32	4.63	9.25	18.3	5.19	11.86
Mean	4.494	2.858	3.704	6.75	3.218	4.426
Std. Deviation	3.712	1.949	3.678	7.322	2.557	5.061
Std. Error of Mean	1.66	0.8715	1.645	3.275	1.144	2.264
Lower 95 % CI of mean	-0.1148	0.4383	-0.8623	-2.342	0.04267	-1.859
Upper 95 % CI of mean	9.103	5.278	8.27	15.84	6.393	10.71

Table 54: *NK T-cells in lymph fluid of group 2, data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

6.3 Influence of compound 4 on the cell populations in blood (group 1 vs group 2)

T-cells

Group 1	Group 2
-42.3 %	13.1 %
-10.9 %	7.6 %
-41.6 %	5.8 %
-30.37 %	-0.4 %
	-4.2 %

Table 55: *Difference between baseline and 3-hour blood values of T-cells in group 1 (n = 4) and group 2 (n = 5).*

Table Analyzed	Col: Unpaired t-test
Column B	group 2
vs.	vs,
Column A	group 1
Unpaired t-test	
P value	0.0018
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 4.887, df = 7
How big is the difference?	
Mean of column A	-31.29
Mean of column B	4.380
Difference between means (B - A) ± SEM	35.67 ± 7.300
95 % confidence interval	18.41 to 52.93
R squared (eta squared)	0.7733
F test to compare variances	
F, DFn, Dfd	4.645, 3, 4
P value	0.1720
P value summary	ns
Significantly different (P < 0.05)?	No
Data analyzed	
Sample size, column A	4
Sample size, column B	5

Table 56: *Unpaired t-test comparing difference between group 1 (n = 4) and group 2 (n = 5) in changes of T-cells.*

NK T-cells

Group 1	Group 2
-1.24 %	-0.25 %
-0.72 %	-0.08 %
-0.98 %	1.13 %
-2.37 %	1.94 %
	0.04 %

Table 57: *Difference between baseline and 3-hour blood values of NK T-cells in group 1 (n = 4) and group 2 (n = 5).*

Table Analyzed	Col: Unpaired t-test
Column B	group 2
vs.	vs,
Column A	group 1
Unpaired t-test	
P value	0.0136
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 3.274, df = 7
How big is the difference?	
Mean of column A	-1.328
Mean of column B	0.5560
Difference between means (B - A) ± SEM	1.884 ± 0.5754
95 % confidence interval	0.5230 to 3.244
R squared (eta squared)	0.6049
F test to compare variances	
F, DFn, Dfd	1.688, 4, 3
P value	0.6957
P value summary	ns
Significantly different (P < 0.05)?	No
Data analyzed	
Sample size, column A	4
Sample size, column B	5

Table 58*: Unpaired t-test comparing difference between group 1 (n = 4) and group 2 (n = 5) in changes of NK T-cell populations.*

Lymphocytes

Group 1	Group 2
-28.4 %	-37.1 %
-28.9 %	-45.2 %
-38.3 %	-36.6 %
-16.5 %	-31.2 %
	-33.8 %

Table 59: *Difference between baseline and 3-hour blood values of lymphocytes in group 1 (n = 4) and group 2 (n = 5).*

Table Analyzed	Col: Unpaired t-test
Column B	group 2
vs.	vs,
Column A	group 1
Unpaired t-test	
P value	0.1073
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t = 1.846, df = 7
How big is the difference?	
Mean of column A	-28.03
Mean of column B	-36.79
Difference between means (B - A) ± SEM	-8.763 ± 4.746
95 % confidence interval	-19.98 to 2.459
R squared (eta squared)	0.3275
F test to compare variances	
F, DFn, Dfd	2.874, 3, 4
P value	0.3342
P value summary	ns
Significantly different (P < 0.05)?	No
Data analyzed	
Sample size, column A	4
Sample size, column B	5

Table 60: *Unpaired t-test comparing difference between group 1 (n = 4) and group 2 (n = 5) in changes of lymphocytes.*

B-cells

Group 1	Group 2
42.3 %	-17.1 %
38.2 %	-5.2 %
40.6 %	-9.5 %
9.5 %	-10.7 %
	-2.8 %

Table 61: *Difference between baseline and 3-hour blood values of B-cells in group 1 (n = 4) and group 2 (n = 5).*

Table Analyzed	Col: Unpaired t-test
Column B	group 2
vs.	vs,
Column A	group 1
Unpaired t-test	
P value	0.0008
P value summary	***
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 5.660, df = 7
How big is the difference?	
Mean of column A	32.65
Mean of column B	-9.060
Difference between means (B - A) ± SEM	-41.71 ± 7.369
95 % confidence interval	-59.13 to -24.29
R squared (eta squared)	0.8207
F test to compare variances	
F, DFn, Dfd	7.927, 3, 4
P value	0.0739
P value summary	ns
Significantly different (P < 0.05)?	No
Data analyzed	
Sample size, column A	4
Sample size, column B	5

Table 62: *Unpaired t-test comparing difference between group 1 (n = 4) and group 2 (n = 5) in changes of B-cells.*

6.4 Influence of compound 4 on the cell populations in lymph (group 2)

Lymphocytes

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 1	96.7	96.4	93.4	94.5	93.8	92.5
Animal 2	96	93.2	93.2	94.5	94.3	92.6
Animal 4	94.6	95.7	95	93.4	94.1	93.7
Animal 5	97.1	94.9	92.9	96.2	95.8	93.8
Animal 6	97	96.2	96.1	93.4	97.4	94.9
Number of values	5	5	5	5	5	5
Minimum	94.60	93.20	92.90	93.40	93.80	92.50
25 % Percentile	95.30	94.05	93.05	93.40	93.95	92.55
Median	96.70	95.70	93.40	94.50	94.30	93.70
75 % Percentile	97.05	96.30	95.55	95.35	96.60	94.35
Maximum	97.10	96.40	96.10	96.20	97.40	94.90
Range	2.500	3.200	3.200	2.800	3.600	2.400
Mean	96.28	95.28	94.12	94.40	95.08	93.50
Std. Deviation	1.033	1.299	1.374	1.147	1.509	0.9874
Std. Error of Mean	0.4620	0.5809	0.6143	0.5128	0.6748	0.4416
Lower 95 % CI of mean	95.00	93.67	92.41	92.98	93.21	92.27
Upper 95 % CI of mean	97.56	96.89	95.83	95.82	96.95	94.73

Table 63: *Lymphocytes in lymph fluid (group 2), data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

Table Analyzed	**Col: Paired t-test**
Column B	3 hours
vs.	vs,
Column A	baseline
Paired t-test	
P value	0.0086
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 4.815, df = 4
Number of pairs	5
How big is the difference?	
Mean of differences (B - A)	-2.760
SD of differences	1.282
SEM of differences	0.5732
95 % confidence interval	-4.352 to -1.168
R squared (partial eta squared)	0.8528
How effective was the pairing?	
Correlation coefficient (r)	0.2022
P value (one tailed)	0.3722
P value summary	ns
Was the pairing significantly effective?	No

Table 64: *Paired t-test, comparing lymphocytes in baseline lymph samples to 3 hours lymph samples of group 2 (n = 5).*

Large cell population

	baseline	15 minutes	30 minutes	1 hour	2 hours	3 hours
Animal 1	2.78	2.89	4.98	3.9	5.42	6.27
Animal 2	2.64	5.4	5.29	3.16	4.72	5.55
Animal 4	2.59	2.44	2.56	3.53	2.59	3.27
Animal 5	2.15	4.16	6.08	2.48	3.19	4.4
Animal 6	1.92	2.04	2.03	3.35	1.33	3.39
Number of values	5	5	5	5	5	5
Minimum	1.920	2.040	2.030	2.480	1.330	3.270
25 % Percentile	2.035	2.240	2.295	2.820	1.960	3.330
Median	2.590	2.890	4.980	3.350	3.190	4.400
75 % Percentile	2.710	4.780	5.685	3.715	5.070	5.910
Maximum	2.780	5.400	6.080	3.900	5.420	6.270
Range	0.8600	3.360	4.050	1.420	4.090	3.000
Mean	2.416	3.386	4.188	3.284	3.450	4.576
Std. Deviation	0.3639	1.379	1.784	0.5259	1.643	1.319
Std. Error of Mean	0.1627	0.6168	0.7978	0.2352	0.7347	0.5900
Lower 95 % CI of mean	1.964	1.674	1.973	2.631	1.410	2.938
Upper 95 % CI of mean	2.868	5.098	6.403	3.937	5.490	6.214

Table 65: *Large cell population in lymph fluid (group 2), data evaluation by performing descriptive statistic including mean, standard deviation, median, quartile, minimum and maximum.*

Table Analyzed	**Col: Paired t-test**
Column B	3 hours
vs.	vs,
Column A	baseline
Paired t-test	
P value	0.0415
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t = 2.961, df = 4
Number of pairs	5
How big is the difference?	
Mean of differences (B - A)	1.960
SD of differences	1.480
SEM of differences	0.6620
95 % confidence interval	0.1219 to 3.798
R squared (partial eta squared)	0.6866
How effective was the pairing?	
Correlation coefficient (r)	0.4559
P value (one tailed)	0.2202
P value summary	ns
Was the pairing significantly effective?	No

Table 66: *Paired t-test, comparing large cells in baseline lymph samples to 3 hours lymph samples of group 2 (n = 5).*

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