ACTIVE COMPONENTS TO REGULATE LIPID SYNTHESIS AND INFLAMMATORY CASCADE IN CULTIVATED HUMAN SZ95 SEBOCYTES

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Geh Wege,
die noch niemand ging,
damit du Spuren hinterlässt.

Antoine de Saint-Exupéry

to Trude Schagen

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ABBREVIATIONS

15-HETE	15-Hydroxyeicosatetraenoic acid
AA	Arachidonic acid
$AACOCF_3$	Arachidonyl trifluoro methyl ketone
AFU	Absolute fluorescence unit
at RA	All trans retinoic acid
Botmo	*Bothrops moojeni*
BRL	Rosiglitazone
BSA	Bovine serum albumin
COX	Cyclooxygenase
$cPGI_2$	Carbaprostacyclin
$cPLA_2$	Cytosolic phospholipase A_2
CRH	Corticotrophin-releasing hormone
DGAT	Diacylglycerol acyltransferase
DHT	5α-dihydrotestosterone
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
EGF	Epidermal growth factor
eLOX3	Epidermal lipoxygenase-3
EM	Emission
EtOH	Ethanol
EX	Excitation
FCS	Fetal calf serum
FDA	Fluorescein diacetate
GF	Gel filtration fraction
GHK	Glycyl-histidyl-lysine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HC	Hydrocortisone
h-EGF	Human epidermal growth factor
HETE	Hydroxyeicosatetraenoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
HPLC	High performance liquid chromatography
HSG	Human sebocyte growth medium
IFN-γ	Interferon-γ
IL-6	Interleukin 6
IL-8	Interleukin 8
LA	Linoleic acid
LDH	Lactate dehydrogenase
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
LTB_4	Leukotriene B_4
MALDI TOF	Matrix assisted laser desorption ionization-time of flight
MFI	Median fluorescence intensity
MMP	Matrix metalloprotease
MS	Mass-spectrometry
Naja m.	*Naja mossambica mossambica*
NDGA	Nordihydroguaiaretic acid
NEP	Neutral endopeptidase
NL	Neutral lipid

NR	Nile Red
NS398	COX-2 inhibitor
PAF	Platelet-activating factor
PAF-AH	Platelet-activating factor acetylhydrolase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E_2
PL	Polar lipid
PLA_2	Phospholipase A_2
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR response element
RP-HPLC	Reversed phase high performance liquid chromatography
RT-PCR	Reverse transcription polymerase chain reaction
SF	Serum free
$sPLA_2$	Secreted PLA_2
$sPLA_2$ IIA	Secreted PLA_2 type IIA
Stau	Staurosporin
SZ95	Immortalized human sebocyte cell line
TGF-α	Transforming growth factor-α
TNF-α	Tumor necrosis factor-α
UV	Ultraviolet

1 SUMMARY

The aim of this work was to search for new active compounds which regulate lipid synthesis *in vitro* in SZ95 sebocytes. More than 200 extracts and fractions derived from plants, microorganisms, *Bothrops moojeni* snake venom as well as peptides were tested in a newly established screening model to identify active ingredients, which act on neutral and polar lipid synthesis in SZ95 sebocytes. The clinical background for this work was the fact that during the ageing process sebocytes reduce lipid production. The relationships between ageing effects and lipid reduction on a molecular level and ways to influence them are not fully identified. For this reason, after identification of active lipid regulating compounds, this work further focused on lipid stimulation in SZ95 sebocytes by *Bothrops moojeni* snake venom gel filtration fractions (Botmo GF).

Botmo GF increased lipid synthesis in SZ95 sebocytes without apparent toxic or apoptotic effects in applied concentrations. Partly purified Botmo GF fractions were identified as fraction with phospholipase (PLA_2) activity (Botmo GF 11-117) and another fraction without enzymatic PLA_2 activity (Botmo GF 11-101). Botmo GF 11-101 (1 µg/ml) enhanced neutral lipid synthesis by up to 150% and polar lipid synthesis by up to 120%. The enzymatically active PLA_2 Botmo GF 11-117 (1 µg/ml) increased synthesis of neutral lipids by up to 310% and polar lipid synthesis by up to 120% compared to untreated SZ95 sebocytes. The present data surprisingly indicate that lipid synthesis stimulation by Botmo GF 11-101 and Botmo GF 11-117 was independent of PLA_2 enzymatic activity in Botmo GF 11 subfractions.
It is hypothesized that SZ95 sebocyte treatment with PLA_2 fractions lead to the production of fatty acids and eicosanoids which activate PPAR. Interestingly, Botmo GF 11-101 was not able to activate any PPAR and Botmo GF 11-117 significantly activated PPARα ($p < 0.001$) in PPARα, δ or γ_2 transiently transfected SZ95 sebocytes.

Phospholipase activates the arachidonic acid (AA) metabolism. AA metabolised with cyclooxygenase (COX) and lipoxygenase (LOX) to prostaglandins as well as leukotrines. To get more knowledge about the lipogenesis pathway, we pre-stimulated SZ95 sebocytes with arachidonic acid and treated sebocytes with cyclooxygenase-2 inhibitor (NS398), LOX inhibitor (NDGA), 5-LOX inhibitor (MK886) and PLA_2 inhibitor ($AACOCF_3$). Interestingly, most of the inhibitors stimulated neutral lipid synthesis in SZ95 sebocytes. Only, PLA_2 inhibitor showed no neutral lipid stimulation.
Additionally, SZ95 sebocytes transiently expressing PPAR were pre-stimulated with arachidonic acid. Treatment with NS398 reduced the level of PPAR isotype activation which, however, remained higher than that of untreated control cells. Since NS398 is a known prostaglandin E_2 (PGE_2) inhibitor, this effect is assumed to be caused by a reduction of PGE_2. The LOX inhibitor NDGA activated all transiently expressed PPAR in SZ95 sebocytes. The epidermal LOX products such as 15-HETE and HPETE may act on PPAR in a non specific manner.

In conclusion, the enzymatically active PLA_2 may activate a pathway of arachidonic acid and mediators which activate lipid synthesis. Botmo GF 11-117 activated PPARα. PLA_2 inactive Botmo GF 11-101 still significantly activated lipid synthesis, while no PPAR activation was measurable. Thus, it is suspected that both *Bothrops moojeni* fractions act via different mechanisms on lipid stimulation in SZ95 sebocytes. However, the exact pathway is still not identified. Botmo GF 11-101 and 11-117 might be interesting tools for the investigation of sebocyte lipogenesis and may be helpful to the development of therapeutic concepts for the treatment of age-related skin dryness.

2 INTRODUCTION

2.1 HUMAN SKIN

The skin is the largest organ of the human body and is organized in three layers: epidermis, dermis and subcutis. The dermis includes epidermal appendages like sebaceous glands, hair follicles and sweat glands. Sebaceous glands and hair follicles form one unit (Figure 1). Each unit consists of bulge, hair bulge and sebaceous gland. Sebaceous glands are found all over the surface of the human body, except on palms, soles and dorsum of the feet. The largest and most active sebaceous glands are located in the face and on the scalp. The main functions of sebaceous glands are sebum production and secretion.
The sebum is composed of different lipids which are produced by sebocytes in the sebaceous glands (for more details see chapter 2.5). Main function of these sebocytes is to produce lipids, which are released upon cell death. The sebum is degraded to pass through the duct to the epidermal surface. Sebum lubricates the skin to protect it against bacterial and fungal infections. A lipid film controls moisture loss and contributes to body odor.

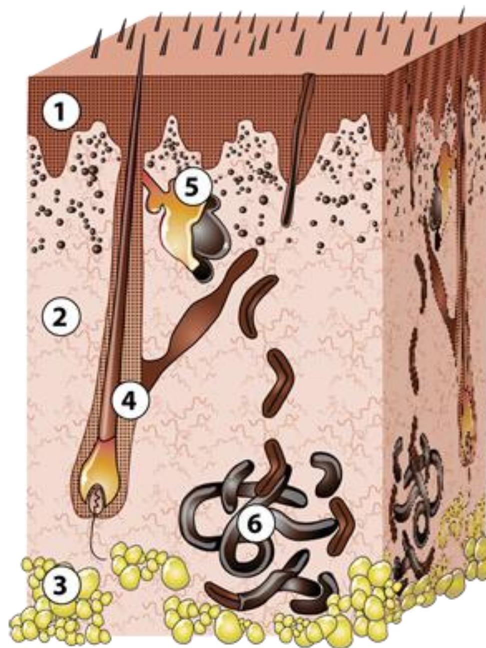


Figure 1 - Schematic illustration of the skin: Skin is composed of three layers; epidermis 1, dermis 2 and subcutis 3. In the inner part of the dermis there is the pilosebaceous unit which consists of: hair bulge 4, sebaceous gland 5, and sweat glands 6.

In more detail, sebocytes are able to express hormones and functional receptors themselves. These receptors bind molecules which initiate pathways for inflammatory cytokines or chemokines, proliferation, differentiation, lipogenesis, and androgen metabolism.

Phenomena in which the pilosebaceous unit which consists of hair bulge, sebaceous gland, and sweat gland is involved are: Skin ageing or disorders like acne, seborrhea, psoriasis and atopic or dry skin.

2.2 CORRELATION BETWEEN SKIN AGEING AND REDUCED SEBACEOUS GLAND ACTIVITY IN HUMAN SKIN

Human life expectancy has increased during the last decades. In the future our society will grow older; the consequences of ageing have begun to gain particular attention. The lifespan of humans is determined by both environmental and genetic factors, and many of the mechanisms identified to increase lifespan are evolutionarily conserved across organisms. Diepgen hypothesizes that life expectancy will grow up to 83 years for woman and 76 years for men in 2025 [28]. It is expected that the group of people aged 65 and more will grow from 17% today to over 30% of Europe's population by 2050.

Skin ageing is a complex process. It is associated with numerous significant gender-related alterations in the lipid profiles secreted by human sebaceous glands.
During ageing, sebaceous glands undergo different structural and functional alterations. Alteration of skin surface lipids is related to the sebaceous gland activity. Gland activity and sebum secretion decrease during the course of ageing (Figure 2).

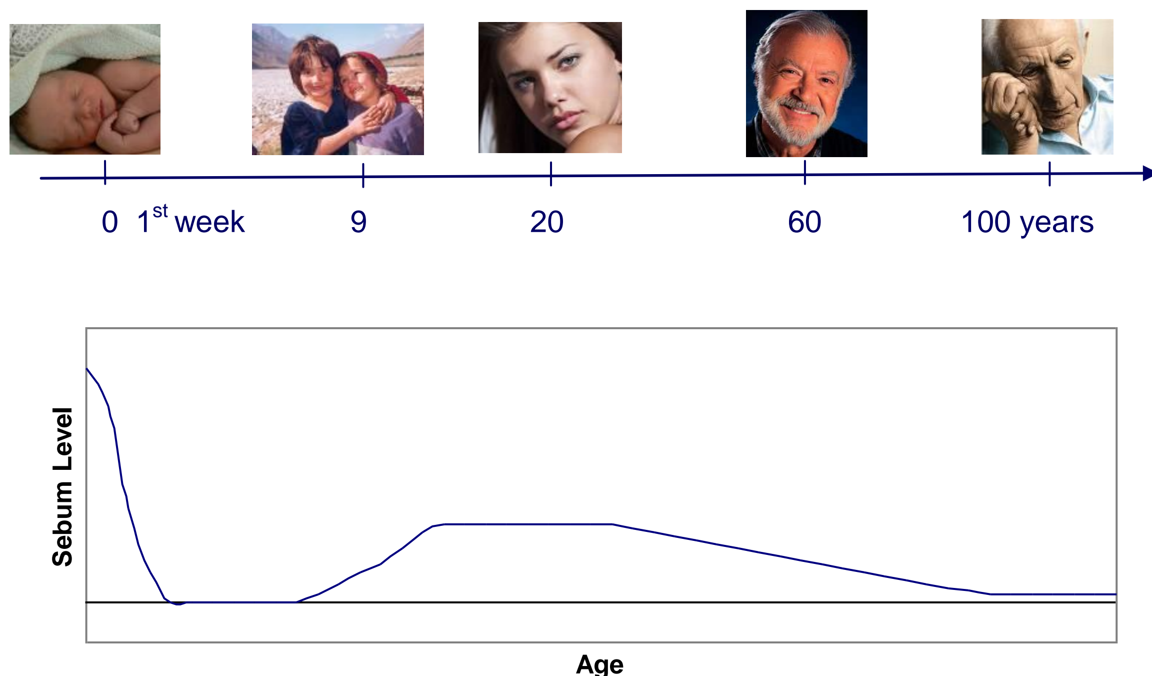


Figure 2 - Sebum secretion level in relation to ageing estimated during life span for women and men [187].

A strong increase in sebum excretion occurs a few hours after birth with a peak during the first week of life, followed by slow decrease thereafter. A next sebum rise takes place at an age of about 9 years and continues up to the age of 20 years, when the adult sebum level is reached. After an age of 60 years sebaceous glands produce reduced levels of lipids. Women show stronger ageing effects with dry skin

after menopause, while men experience a minimal sebum decrease, usually after the age of 80 years.

The number of sebaceous glands remains approximately unchanged throughout life; however their size increases with age [157]. Over time, sebaceous glands reduce sebum secretion. As a result of this skin slowly loses its ability to renew itself and becomes thinner and drier. Dryness is one important characteristic of aged skin. Changes in the connective tissue reduce the skin's strength and elasticity. Additionally, aged skin repairs itself more slowly than younger skin. Figure 3 illustrates the visible changes occurring during skin ageing on the less sun exposed inner arm.

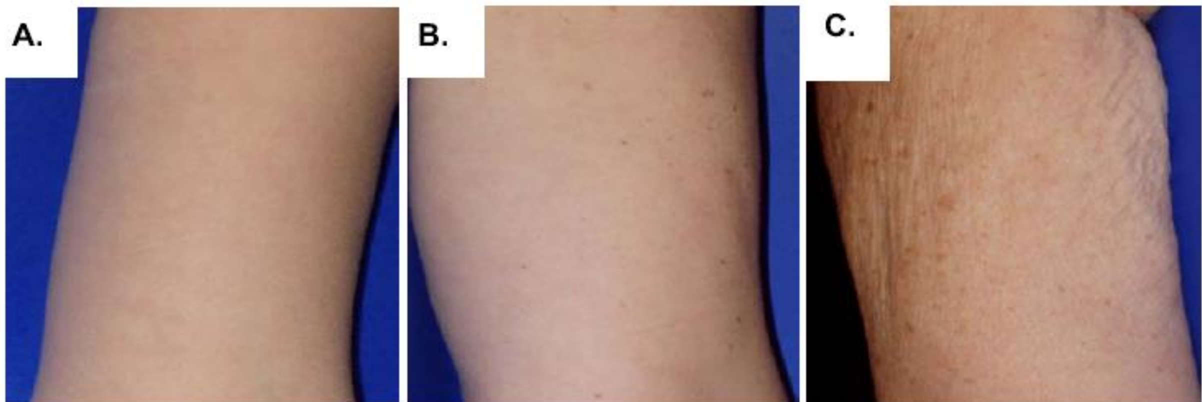


Figure 3 - Aged skin in the inner less UV exposed area of the arm. Photos are taken from a 10- (A), 45- (B) and 70- (C) year old person [88].

2.3 SEBACEOUS GLANDS AND DEVELOPMENT OF SEBOCYTES

Epidermal progenitor cells in skin give rise to multiple lineages: hair follicle, sebaceous gland, and overlying epidermis. The multi-potent stem cells reside in the bulge region of the hair follicle. These stem cells transform into keratinocytes (epidermis) as well as into associated structures such as sebaceous glands (sebocytes) and dermal papilla (dermal papilla cells) (Figure 4)

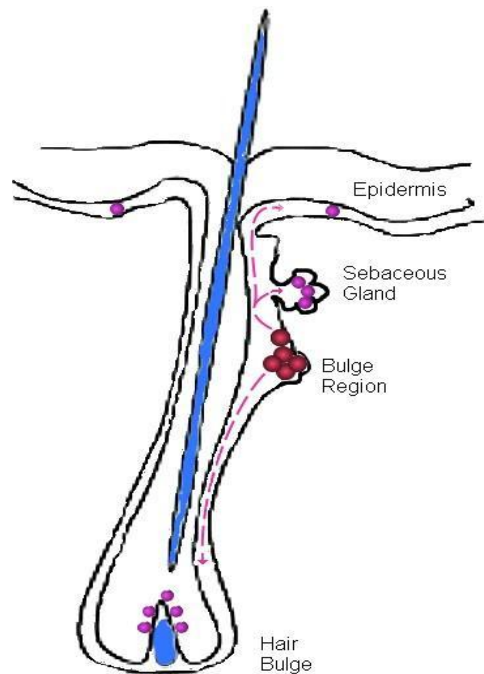


Figure 4 - Model follicle showing the development of sebocytes, dermal papilla cells (hair bulge) and keratinocytes (epidermis). The stem cells reside in the bulge region of the hair follicle and differentiate to sebocytes in the sebaceous glands [128]

Many of the molecules which regulate epidermal self renewal and differentiation have been identified. Several molecular networks and signaling pathways are important in balancing epidermal growth and differentiation. Some of these key compounds are NF-κB, Wnt/β-catenin, sonic hedgehog/patched, p63, 14-3-3σ, α-catenin and β1-integrin [172]. However the signals involved in sebocyte development are poorly understood.

The level of β-catenin regulates lineage selection by stem cell progeny in mammals. High levels of β-catenin stimulate the formation of hair follicles while low levels lead to creation of epidermis and sebaceous glands. Intracellular signaling molecules like transcription factor 3 (Tcf3) and Lymphoid-enhancing factor-1 (ΔNLef-1), a DNA binding molecule, control lineage differentiation. Overexpression of ΔNLef-1 blocks β-catenin signaling. Niemann et al. propose that ΔNLef-1 and Indian Hedgehog (IHH) cooperate to control proliferation and differentiation of sebocyte progenitors [104]. Sonic hedgehog (SHH) is a signaling molecule for the regulation of progenitor cells of hair lineage differentiation and proliferation. SHH is not required for terminal differentiation of the hair lineage, which is controlled by the Wnt signaling pathway [42, 105]. Inhibition of Wnt target genes using a dominant negative ΔNLef-1 promotes sebocyte development while inhibiting differentiation of the hair lineage [97, 103] (Figure 5).

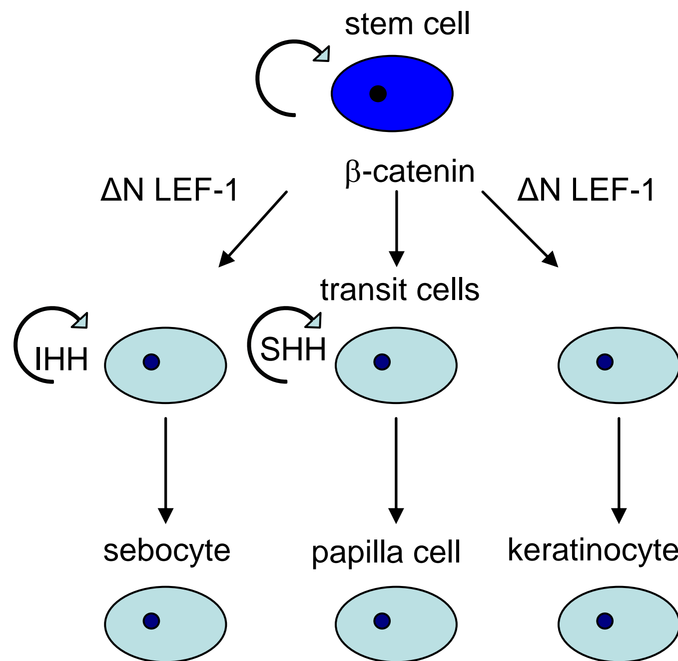


Figure 5 - Model of interaction between ΔNLef-1, β-catenin and hedgehog signaling in epidermal stem and progenitor cells. The high level of β-catenin is necessary to develop papilla cells, ΔNLef-1 blocks β-catenin for sebocytes, keratinocytes development [104].

The hedgehog pathway and other molecules are suggested to be implicated in sebocyte development, including activation of c-Myc, PPAR and COX-2 [5]. Activation of c-Myc stimulates epidermal proliferation and induces differentiation of sebocytes within the interfollicular epidermis [17].

2.4 HUMAN SEBACEOUS GLAND ISOLATION AND SEBOCYTE CULTIVATION

Selective cultivation of sebocytes *in vitro* is essential for a better understanding of the pathophysiology of human skin, for drug development and investigation of drug pharmacokinetics. Data on sebaceous gland physiology initially emerged from the use of animal sebaceous gland models. Animal glands produce a higher amount of sebocytes than human sebaceous glands. The composition of intracellular lipids from hamster sebaceous gland-derived cells was very similar to human sebocytes, except that the hamster sebocytes did not contain any squalene and wax [62].
In 1966, Kellum first described the isolation of sebaceous glands from human skin. However his experiments did not allow the maintenance of viable sebaceous glands *in vitro* [70]. In 1982, Karasek and Charlton reported the cultivation of human sebocytes, obtained from dermal slices rich in sebaceous gland tissue, to be used as a model for the investigation of human sebaceous glands [66]. Kealey et al. and Xia et al. introduced the maintenance of the sebaceous gland *ex vivo* and the cultivation of sebaceous gland cells *in vitro*, respectively [68, 174]. Several modifications of the technique of Xia et al., including sebocyte isolation and cultivation, have facilitated the reproducible cultivation of human sebocytes *in vitro* [174, 201].
However, human sebocytes are predisposed to differentiate by accumulating neutral fat droplets until they burst and die. Therefore, adequate cell numbers for large scale

experiments can only be obtained from multiple donors, while prolonged experiments are hindered by the short life span of the cells. Normal human sebocytes can only be grown for 3 to 6 passages. To overcome this problem, Zouboulis and colleagues cultured human facial sebocytes from an 87-year-old woman and transfected them with the Simian Virus-40 large T antigen. This gave rise to the immortalized cell line SZ95, allowing prolonged survival of the cells *in vitro* [199]. Several studies have shown that SZ95 sebocytes retain major characteristics of normal human sebocytes, such as differentiation with increased cell volume and lipid synthesis as well as subsequent apoptosis, expression of characteristic origin- and function-specific proteins of human sebaceous glands, and expected biological response to androgens and retinoids [173, 199]. Differentiation markers for fully differentiated sebocytes are keratin 7, keratin 19 and epidermal membrane antigen [201].
Since the first development of a standardized human sebaceous gland cell line (SZ95) [199], sebaceous gland research has experienced a new area with numerous research groups including sebocytes in their scope, whereas animal models have almost been abandoned. With the help of human sebocyte cultures research on acne and other sebaceous gland associated diseases is getting closer to the human *in vivo* situation [185].
In 2003, Thiboutot and colleagues applied the transfection system used by Zouboulis et al. to develop a second immortalized human facial sebaceous gland cell line, termed SEB-1. SEB-1 was established from sebaceous glands from normal skin of the predauricular area of a 55-year-old male [159]. Like SZ95 sebocytes, SEB-1 sebocytes express characteristic sebaceous gland proteins and induce lipid droplets. Gene array studies showed that genes characteristic of sebaceous glands and those involved in lipid and steroid metabolism were expressed in SEB-1 sebocytes [185].
A third immortalized sebaceous gland cell line, Seb-E6E7, has been generated from adult human facial skin [81]. Human sebocytes were immortalized by introduction of HPV16 E6 and E7 genes. Seb-E6E7 sebocytes were transduced by co-culture with mitomycin C-treated packaging cells in the presence of 3T3-J2 cells. Seb-E6E7 sebocytes, like SZ95 sebocytes, express both keratin 7 and involucrin [81].

2.5 SEBUM AND SEBOCYTE LIPIDS

One of the main functions of sebocytes is sebum production. The major role of sebum and its lipid content is skin surface protection from environmental influences like UV, hazardous chemicals and microbes.
Human sebum is a lipid mixture composed of glycerides, wax esters, squalene, cholesterol, cholesterol esters and free fatty acids. The composition of the intracellular lipids from mammalian sebaceous gland derived cells is similar to human sebaceous gland derived cells, except that mammalian sebocytes do not contain any squalene [62, 112]. Wax esters and squalene are only synthesized by human sebocytes [112].
Fatty acids and fatty alcohols are natural and direct precursors of wax esters. A fatty acid transport protein ($FATP_4$) is strongly expressed by sebaceous glands. This trans-membrane protein enhances the uptake of long chain fatty acids into sebocytes [139]. As a consequence, wax ester biosynthesis is raised in human sebaceous gland cells [112]. The ability of sebocytes to synthesize wax esters correlates with the activity of β-oxidation in these cells. Linoleic acid undergoes β-oxidation to γ-linoleic acid. It seems that β-oxidation of linoleic acid is specific for sebocytes and associated with their differentiation [112].

Ge et al. identified Δ-6 desaturase/$FADS_2$ as the major fatty acid desaturase in human sebaceous glands, which synthesizes very long chain fatty acids. Desaturases add double bonds between carbon 6 and 7 of linolenate and α-linolenate to generate the polyunsaturated fatty acids γ-linolenate and stearidonate. Palmitate is elongated to the monounsaturated fatty acid sapienate. Sapienic and sebaleic acids are the predominant fatty acids, which are unique in humans compared to other hair bearing mammals [45, 102, 112, 156].

Table 1 - Lipid composition of sebaceous glands and cultured sebocytes in (%)

	Adult sebaceous glands	Freshly isolated sebaceous glands	Primary sebocytes
Squalene	9.3 – 12	20 - 29	2
Sterol/wax ester	24 - 27.1	6.5 - 7.1	4
Triacyl glycerides	23 – 41	23 - 54	40
Free fatty acids	14 – 27	0.8 - 1.2	20
Cholesterol	1.4 - 4.2	1.5 - 2.1	2
Diglycerides	-	2.5	7
Others	2 – 20	38	25

Data from Nikkari, Cassidy and Ridden [18, 106, 127, 200]

In vitro sebocytes synthesize less squalene, wax esters and cholesterol esters than sebaceous glands *in vivo*, but they are still capable of producing the natural lipid composition [133, 200]. Squalene may serve as a marker for terminal sebocyte differentiation [200] (Table 1).

Table 2 - Lipid content in treated and untreated hamster sebocytes in (%)

	Untreated	Oleic acid treated
Squalene	33	49
Phospholipids	59.7	30.1
Free cholesterol	4.1	-
Free fatty acids	0.97	12.2
Triglycerides	0.58	6.0

Analysis of extracted hamster sebocyte lipids revealed differences in lipid composition after addition of oleic acid to the culture medium [100]. After oleic acid treatment the concentration of squalene, fatty acids and triglycerides was largely increased and phospholipids concentration was decreased [100] (Table 2).

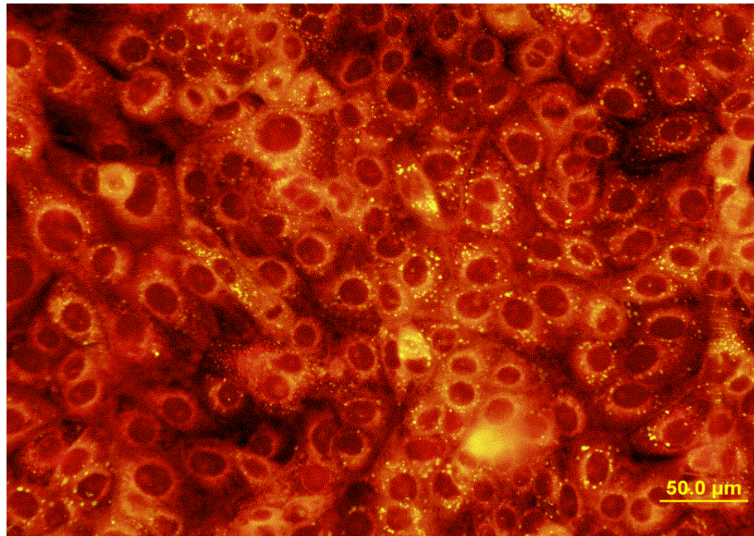


Figure 6 - Nile Red fluorescence staining of SZ95 sebocytes: Polar lipids (red) such as phospholipids containing organelles and membrane lipids are visualized at 540 / 620 nm. Unpolar or neutral lipids (yellow) such as triglycerides and wax esters are visualized at 485 / 528 nm [138].

Sebocyte lipids can be visualized with Nile Red or Oil Red staining (Figure 6) [50, 199]. Nagai found that cultured hamster-cloned and rat primary sebocytes contain membrane vesicles, defined as sebosomes, containing squalene condensed lipid particles and enriched with histone H_3, a cationic protein [100]. The presence of these vesicles suggests a cell function associated with secretion of antibacterial proteins, sterol regulation and a possible role in protecting the skin surface [100].
Additionally, perilipin A has been shown to be present on the surface of intracellular lipid droplets in differentiated hamster sebocytes *in vitro* [2]. Perilipin A is a protein in mammals which envelops lipid droplets in fat cells and protects the cell from the fat burning enzyme lipase.

2.5.1 INTRACELLULAR LIPIDS IN SEBOCYTES

In sebocytes, intracellular lipids are stored in lipid droplets. Neutral lipids play a major role as moisturizers and in the protection of human skin. Lipid synthesis in SZ95 sebocytes is increased by androgens and fatty acids, whereas it is inhibited by hydrocortisone, retinoids, and estrogens [191].

The free fatty acids in sebum have been considered to participate in the inflammatory process. Linoleic acid and arachidonic acid are necessary for the first steps in the production of proinflammatory cyclooxygenase products, but they stimulate lipid synthesis in sebocytes as well. Furthermore, arachidonic acid up-regulates the secretion of IL-6 and IL-8 in sebocytes [4]. Arachidonic acid is an activating ligand of peroxisome proliferator-activating receptors (PPAR) which play a role in regulating lipogenesis in sebocytes [196].
In SZ95 sebocytes treated with arachidonic acid, lipid accumulation and apoptosis are enhanced. Terminal differentiation and apoptosis are two different programmed cell events, which both result in cell death. Terminal differentiation of sebocytes is a part of lipid synthesis. It begins with the accumulation of lipid droplets in the cytoplasm and results in apoptosis induction and bursting of the cell [173].

There is evidence that epidermal growth factor and 1α-25-dihydroxyvitamin D_3 act as suppressors in the regulation of lipogenesis in hamster sebocytes *in vitro* [134]. Epidermal growth factor, transforming growth factor-α and fibroblast growth factor have mitogenic activity on hamster sebocytes and act as anti lipogenic factors. Moreover, it is likely that the formation of intracellular lipid droplets is independent of cell proliferation in hamster sebocytes [3]. The most understood compounds that down regulate lipid synthesis *in vitro* are the retinoids, such as isotretinoin (13-cis retinoic acid), tretinoin (all-trans retinoic acid), 9-cis retinoic acid, etretinate, and acitretin. Isotretinoin is the most potent component which suppresses sebum production and significantly decreases lipogenesis, thereby reducing proliferation and differentiation of sebocytes [108, 192, 193]. Isotretinoin and tretinoin reduce proliferation of cultured sebocytes in a dose- and time-dependent manner; however the complete mechanism of action remains unknown. Marked decreases in wax esters, a slight decrease in squalene and a relative increase in cholesterol level were measured.
Hydrocortisone stimulates the proliferation of sebocytes in a dose-dependent manner. Cortisone reduces lipid production and anti-inflammatory effects when the cells are grown to confluency [200].

Table 3 - Neutral lipid synthesis regulating compounds

Compounds	Effect on neutral lipid synthesis	Reference
Androgen	↑	[90, 131, 134]
Arachidonic acid	↑	[4]
Linoleic acid	↑	[90]
1α-25-Dihydroxyvitamin D_3	↓	[134]
All-trans retinoic acid	↓	[108, 192, 193]
Epidermal growth factor	↓	[134]
Estrogens	↓	[187]
Hydrocortisone	↓	[200]
Transforming growth factor-α	↓	[3]

Legend: ↑ up regulation ↓ down regulation

2.5.2 CELL MEMBRANE LIPIDS

There are three classes of cell membrane lipids: phospholipids, cholesterol and glycolipids (Table 4). Human cells contain large amounts of cholesterol and a wide spectrum of different phospholipases with diverse functions. There are four main types of phospholipids: phosphatidylcholin, sphingomyelin, phosphatidylserine and phosphatidylethanolamine. Half of the lipid content in most membranes is a mixture of these four phospholipids.

Table 4 - Functions of polar membrane lipids

Membrane lipids	Functions	Reference
Phospholipids	• Cell proliferation • Membrane homeostasis • Membrane repair through deacylation/reacylation • Induction of inflammation • Barrier function	[175]
Cholesterols	• Prevention of hydrocarbon crystallization and phase shifts in the membrane • Required for permeability barrier homeostasis • Membrane structure • Regulation of ion pumps	[91, 175]
Glycolipids	• Barrier function • Interaction between cells and cell surroundings	[59]

Cholesterol prevents crystallization of hydrocarbons and phase shifts in the membrane. It immobilizes the first few hydrocarbon groups of the phospholipid molecules, thus making the lipid bilayers less deformable and decreasing their permeability to small water-soluble molecules.
Glycolipids are located in the outer layer of the membrane bilayers. Their sugar groups are exposed on cell surface. Assumedly glycolipids are involved in interactions between the cell and its surrounding. Glycolipids seem to be functionally linked to cholesterol [59].

2.5.3 PHOSPHOLIPASE IN THE EPIDERMIS

The presence of phospholipase A_2 (PLA_2) activity was demonstrated several years ago, however the precise location of the different PLA_2 in the epidermis and its appendages has still to be determined. Further studies have shown that these enzymes are expressed in various layers of the epidermis. Phospholipids in eukaryotic membranes do not only play a structural role, but are also involved in many metabolic processes.
Phospholipase A_2 (PLA_2) catalyzes hydrolysis of the sn-2 fatty acid substituent from glycerophospholipid substrates to yield a free fatty acid, e.g., arachidonic acid and a 2-lysophospholipid (Figure 7) [152] that have intrinsic mediator functions [125] and can initiate synthesis of other mediators, such as prostaglandins, leukotrienes, epoxy-eicosatrienoates, and platelet-activating factor (PAF) [11].
Haas et al. and Gurrieri et al. observed different PLA_2 expressions levels in skin cells. The specifically secreted phospholipase A_2 expression profile in the skin suggests a distinct function for each enzyme in the epidermis. The PLA_2 expression from keratinocytes and primary keratinocytes from healthy patients compared to psoriasis patients were investigated [51, 54]. In short, immunofluorescence studies showed that PLA_2 IB, IIF, and X were predominantly expressed in suprabasal layers, whereas $sPLA_2$ V and IID were detected in the basal and spinous layers. Secreted PLA_2 IIA

was weakly identified and $sPLA_2$ IIE and XIIA were not detectable. In differentiated primary keratinocytes the expression of $sPLA_2$ IB, IIF, X was increased, whereas $sPLA_2$ V and IID were markedly decreased. In psoriatic skin $sPLA_2$ X was dramatically down-regulated in the epidermis, while increased amounts of $sPLA_2$ X, IIA, IID and IB appeared in the dermis [54].

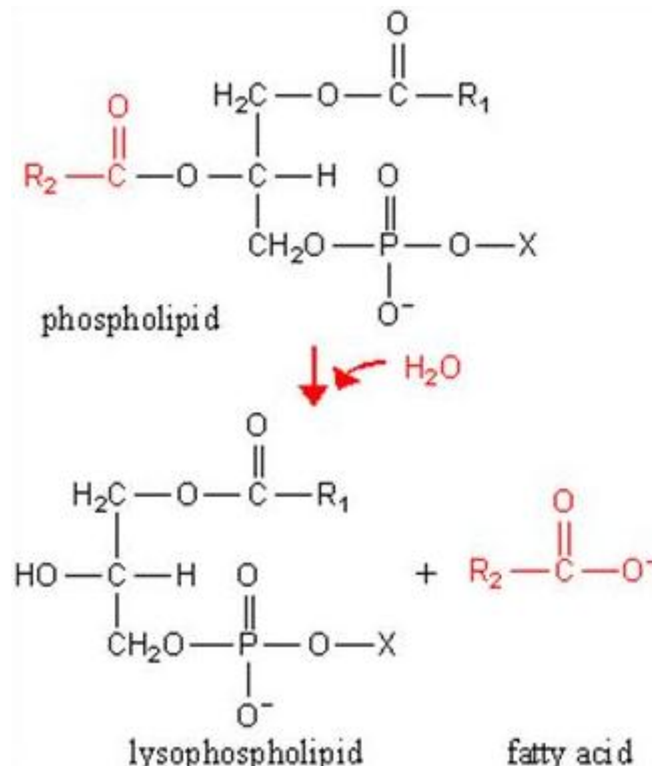


Figure 7 - Phospholipase metabolism: Phospholipases A_2 are enzymes that catalyze the hydrolysis of glycerophospholipids at the sn-2 position, generating free fatty acids and lysophospholipids [35].

At present, the PLA_2 family consists of 12 groups. PLA_2 are involved in many physiological processes such as barrier function, eicosanoid production, and inflammation. They are connected to inflammatory diseases of the skin like psoriasis, eczema, and atrophy. Their differential localization suggests different roles for each PLA_2 in skin physiology and during inflammation [96].

The secreted PLA_2 ($sPLA_2$) group, in which 10 isozymes have been identified, consists of low-molecular-weight, Ca^{2+}-requiring, secreted enzymes that are known to be involved in a number of biological processes, such as modification of eicosanoid generation, inflammation, host defense and atherosclerosis. The cytosolic PLA_2 ($cPLA_2$) group consists of three enzymes, among which $cPLA_{2\alpha}$ plays an essential role in the initiation of arachidonic acid metabolism. Intracellular activation of $cPLA_{2\alpha}$ is tightly regulated by Ca^{2+} and phosphorylation. The Ca^{2+} independent PLA_2 group contains two enzymes and may play a major role in membrane phospholipid remodeling. Platelet-activating factor acetylhydrolase (PAF-AH) represents a unique group of PLA_2 that contains four enzymes exhibiting unusual substrate specificity toward PAF and/or oxidized phospholipids [99].

2.6 EXPRESSION OF PROTEINS AND HORMONES BY SEBOCYTES AND THEIR BIOLOGICAL ACTIVITIES

Human skin is an endocrine organ which synthesizes hormones. Hormones are biochemically active messengers and coordinate activity in different cell types in multi-cellular organisms. The cells of the skin are able to metabolize, activate, and inactivate hormones. Human sebocytes themselves produce corticotrophin-releasing hormone (CRH), androgens, estrogens, all trans retinoic acid, cortisol, calcitriol (vitamin D_3) and eicosanoids [183, 187]. Sebocyte proliferation, differentiation and lipid synthesis (accumulation of neutral lipids) are controlled by this complex endocrine hormone system. CRH activates proliferation and induces lipid synthesis in sebocytes.
Androgens affect several functions of human skin, such as sebaceous gland growth and differentiation. In cell culture, androgens promote sebocyte differentiation as well as proliferation and show a lipogenic activity [134].
Estrogens, cortisol, and all trans retinoic acid down regulate differentiation, cell proliferation and intracellular accumulation of neutral lipids. The effects of estrogens can be explained by inhibition of gonadotropin secretion or by enhancement of testosterone binding. Cortisol also influences lipid metabolism, wound healing, and relieves stress.
Calcitriol has various cellular functions including anti proliferative effects on SZ95 in logarithmic phase of cell growth and differentiation [141]. In addition, 1α-25-dihydroxyvitamin D_3 decreases lipid synthesis in sebocytes [134].

2.7 HORMONE RECEPTORS AND THEIR PROPERTIES IN HUMAN SEBOCYTES

Sebocytes express receptors for peptide hormones and neurotransmitters, which are mostly arranged on the cell surface. Steroid and thyroid receptors are found in the cytoplasm and nuclear compartment. Hormones (agonists) bind to the receptors with high affinity. This in turn triggers further pre-defined reactions (Figure 8).

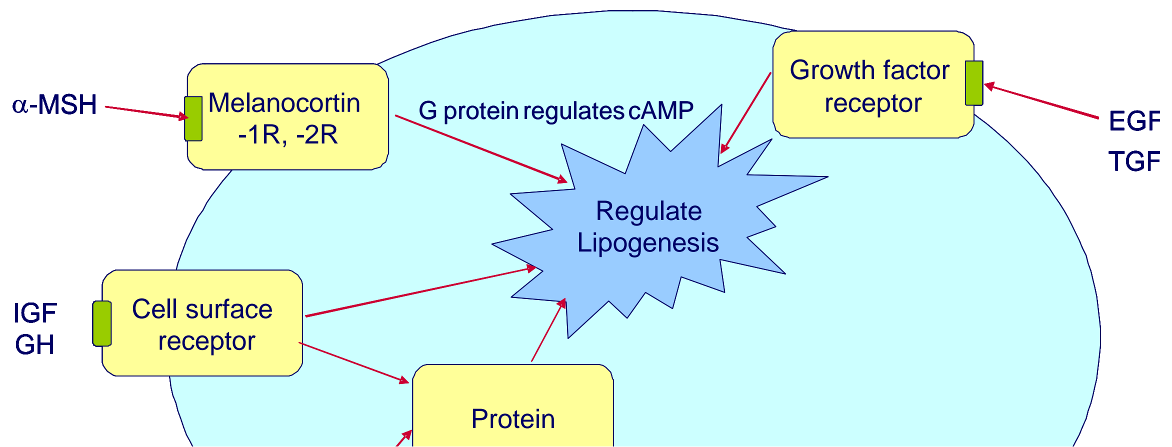


Figure 8 - Interaction between membrane and nuclear receptors in human sebocytes: Their influence on lipid accumulation is displayed. The release of various hormones, growth factors and mediators of inflammation can regulate lipogenesis in sebocytes. Activator (green), receptor (yellow), corticotrophin-releasing hormone (CRH), melanocyte-stimulating hormone-α (MSH-α), growth hormone (GH), insulin-like growth factor (IGF), epidermal growth factor (EGF), transforming growth factor-α (TGF-α), peroxisome proliferator-activated receptor (PPAR) [189].

Extracellular receptors regulate three kinds of intercellular communication pathways: The endocrine signaling pathway transports hormones to their receptors, the paracrine pathway absorbs, immobilizes and catabolizes local chemical mediators and the synaptic signaling pathway acts on the post synaptic part of cells. All cells in the human body express a defined combination of different receptor proteins which can interact with complementary signaling molecules to activate a characteristic cell response.

Signal molecules can be classified according to their solubility in water. Most hydrophilic molecules bind to receptors on the cell surface, whereas hydrophobic molecules like steroid hormones pass through the plasma membrane of the cell and activate nuclear receptors in the cell cytoplasm.

Table 5 - Receptors and binding affinity of sebocytes

Group	Receptors	Binding affinity	Reference
Extra cellular cell surface	CRH-1R,-2R, MC-1R, MC-5R, μ-opinate-R, IGF-1R, GHR, Substance P, CGRPR,VPAC-2, NYR,	Integral membrane proteins bind to an extracellular domain and activate a tyrosine specific protein kinase in the cytoplasm	[14, 143, 186, 189, 198]
Nuclear receptor	AR, ERα, ß, PRA, PRB, RAR, RXR, PPARα, β/δ, γ_1, γ_2	They are activated by distinct lipophilic small molecules such as glucocorticoids, estrogens, androgen, progesterone, retinoid and fatty acid derivatives.	[8, 186]

This table summarizes all known receptors which are active in human sebocytes and their binding affinity: Corticotrophin-releasing hormone 1-receptor (CRH-1R), corticotrophin-releasing hormone 2-receptor (CRH-2R), melanocortin-1 receptor types (MC-1R), melanocortin-5 receptor types (MC-5R), μ-opiate receptor (μ-opiate-R), vasoactive intestinal polypeptide receptor type 2 (VPAC-2), neuropeptide Y receptor (NYR), calcitonin gene-related peptide receptor CGRPR, insulin-like growth factor receptor (IGFR), growth hormone receptor (GHR), androgen receptor (AR), estrogens receptor-alpha (ERα), estrogens receptor-beta (ERß), substance P, retinoid acid receptor (RAR), retinoid x receptor (RXR), peroxisome proliferator-activated receptor (PPARα, β/δ, γ), progesterone receptor A (PRA), progesterone receptor B (PRB).

2.7.1 EXTRACELLULAR RECEPTORS OF SEBOCYTES

Water soluble signaling molecules (neurotransmitters, protein hormones, and growth factors) as well as some soluble lipid signaling molecules bind specific receptor proteins on the surface of target cells with a high affinity. The receptor then transforms the extracellular signal into an intracellular response which alters the behavior of the target cell.

Neuropeptides do not only act in the central nervous system, they are also active and expressed in skin cells. Neuropeptides have a neuroendocrinic regulatory effect on sebocytes and are the link between emotional stress and acne [15, 187, 198]. Corticotrophin-releasing hormone (CRH) and its receptors are expressed in sebaceous glands. The expression of CRH, CRH-binding protein, and CRH receptors (CRH-R) in SZ95 sebocytes was investigated at the mRNA and protein level [198]. CRH is an important autocrine hormone, with a pro differentiation and lipid synthesis inducing activity. CRH enhances mRNA expression of Δ5-3β hydroxysteroid dehydrogenase and formation of testosterone in human sebocytes [41, 198]. CRH directly induces lipid synthesis and enhances mRNA expression of Δ5-3β-hydroxysteroid dehydrogenase, an enzyme which converts dehydroepiandrosterone to testosterone in human sebocytes. Testosterone and growth hormone (GH) antagonize CRH through modulation of its receptor. GH switches the predominant CRH receptor-1 in SZ95 sebocytes to CRH receptor-2 [101, 194].

Melanocortin receptors (MC-1R, MC-5R) and their ligands melanocyte-stimulating hormone (MSH) and adrenocorticotrophin hormone (ACTH) have been shown to

influence physiological functions of cells and organs, including exocrine glands. MC-Rs are located at the cell surface of sebocytes. MC-1R in SZ95 sebocytes and in primary sebocytes was first described by Böhm et al. [189]. MC-1R is expressed to higher levels than MC-5R. In SZ95 sebocytes, MC-5R expression is not detectable [15]. In 2006, Zhang examined the expression of MC-5R in human sebocytes *in vivo* and *in vitro*. MC-5R was only detected in differentiating, lipid-laden sebaceous glands but not in basal undifferentiated cells. Zhang et al. suggest that MC-5R is a marker of human sebocyte differentiation [180]. The immunomodulatory actions of α-melanocyte stimulation include regulation of expression and secretion of chemokines, down regulation of proinflammatory signal induced NF-κ3 activation and adhesion molecule expression PGE_2 synthesis as well as induction of IL-10 [15].

The human sebocyte is a direct cellular target of α-melanocyte-stimulating hormone (α-MSH), which appears to regulate lipogenesis and the production of proinflammatory cytokines. It is unclear, whether α-MSH or adrenocorticotropin (ACTH) is produced by sebocytes. Experiments in animals showed a stimulatory effect of α-MSH and ACTH on sebum production. However, α-MSH in human cultured sebocytes inhibited the IL-1β induced release of IL-8 [194]. A lipogenic effect on sebocytes also occurs after β-endorphin stimulation [194].

The μ-opiate receptor is expressed in human SZ95 sebocytes and sebaceous glands [14] and binds β-endorphin. This stimulates lipogenesis and specifically increases the amount of C16:0, C16:1, C18:0, C18:1 and C18:2 fatty acids to a similar extent as linoleic acid stimulation of sebocytes [14].

Insulin-like growth factor receptor (IGF-R) is expressed on the sebocyte cell surface and can be activated by IGF or a high dose of insulin [86]. *In vitro* studies have shown IGF-1 dependent stimulation of sebocyte proliferation and differentiation especially in combination with growth hormone (GH) [25]. IGF enhances lipid production in human SZ95 sebocytes in a dose-dependent manner.

The activation of IGF-1R induces lipogenesis in SEB-1 cells via both the sterol response element binding proteins dependent and independent pathway. Sterol response element binding proteins are nuclear transcription factors and regulate the synthesis of cholesterol and fatty acids. They are produced as precursors stored in the endoplasmic reticulum [154].

The growth hormone also up-regulates sebocyte differentiation and augments the effect of 5α-dihydroepiandrosterone on sebum synthesis [169]. Growth hormone binds to a membrane bound receptor and thus has a direct effect on sebocytes. Its activation is mediated by IGF [25].

Substance P can be released through stress; it promotes the development of cytoplasmic organelles in sebaceous cells and stimulates sebaceous germinative cells. It also increases the size of individual sebaceous cells and the number of sebum vacuoles for each differentiated sebaceous cell, all of which suggest that substance P promotes both the proliferation and the differentiation of sebaceous glands [165]. Neutral endopeptidase, an enzyme that degrades substance P and thus reduces the pro-inflammatory effect of substance P, is highly expressed in the sebaceous glands of acne patients. Finally, an increase in the number of nerve fibers is noted around the sebaceous glands of acne patients and sometimes inside the glands. The latter is related to an increased expression of nerve growth factor in acne prone sebaceous glands [13]. Substance P often co-localizes with calcitonin gene related peptide [155]. Calcitonin gene related peptide signaling in keratinocytes is involved in c-AMP induction and Ca^{2+} mobilization [117]. IL-6 produced by mast cells induces production of nerve growth factors in sebocytes [14]. Neuropeptides are transmitter substances produced by neuronal and non neuronal cells of the skin.

Seiffert et al. showed that the expression of calcitonin gene related peptide receptors, vasoactive intestinal polypeptide receptors and neuropeptide Y receptors are present in human sebocytes; their agonists activate cytokine production [143].

2.7.2 NUCLEAR RECEPTORS OF SEBOCYTES

Hydrophobic ligands are able to pass cell membranes to activate receptor proteins or nuclear receptors in the cytoplasm.
Azzi et al. show gender differences in mouse sebocytes. Androgen receptors (AR) in male mice are exclusively localized in the nuclei of basal and mature sebocytes. In females there are present in a lower level in both the nuclei and the cytoplasm [8]. ARs are ligand activated intracellular transcription factors, are stabilized by ligand binding, which in turn leads to up regulation of lipid synthesis in sebocytes. Five enzymes are involved in activation and inactivation of androgens. Dehydroepiandrosterone sulfate (DHEA-S) is metabolized with steroid sulfatase to dehydroepiandroestrone (DHEA). DHEA and androtestosterone are converted to testosterone and later to 5α DHT by intracellular enzyme 5α-reductase in human skin. Sebocyte studies of Akamatsu et al. show a dose-dependent induction of sebocyte proliferation by testosterone treatment [1] and no effect on lipid stimulation [199]. Additional investigations by Rosenfield present evidence that the effect of testosterone is regulated by PPAR ligands [130]. All PPAR isotype are present in sebocytes and regulate multiple lipid metabolic genes [19, 87, 131]. In a study of Makrantonaki testosterone leads to a significant increase of polar lipid production. This lipid production can be used as a marker for sebocyte proliferation.
Estrogens play an important role in tissue of both sexes. Estrogen receptor β (ER-β) is expressed in basal and partially differentiated sebocytes. Estrogen receptor α (ER-α) and AR are both expressed in basal and early differentiated sebocytes [162].
Estrogens, estradiol and testosterone are formed by oxidative reduction of 4-androsten-3, 17-dion. An experiment with 17β sterol sebocyte treatments show an effect on polar lipid production but no lipid-stimulating effect on neutral lipids in sebocytes. Other *in vitro* results demonstrate the influence of estrogens on the biological activity in sebaceous glands [53].
Progesterone receptor (PR) was found in nuclei of basal sebocytes in sebaceous glands [114]. There were no significant differences in PRA, PRB and AR immunoreactivity between non-pathological sebaceous gland and its neoplasm [67].

Two retinoid receptors are expressed in sebocytes. The retinoid acid receptor (RAR) modulates cell proliferation and the retinoid x receptor (RXR) influences cell differentiation. The natural ligands for RAR and RXR have been identified as all trans retinoic acid and 9-cis retinoic acid, respectively. 13-cis retinoid inhibits proliferation in sebocyte cell line SZ95. It was found that 13-cis retinoic acid is metabolized to all trans retinoic acid [130]. All trans retinoic acid binds to retinoic acid binding protein II, which in turn binds and activates nuclear receptor RAR [101].
The retinoid x receptor is a ligand dependent transcriptional regulator. RXR is known to form a heterodimer with PPAR. Several gene receptor studies have shown that either RXR ligand or PPAR ligand can activate the RXR-PPAR heterodimer [130].

RXR agonists stimulate sebocyte differentiation and proliferation. Kim reports dose-dependent effects of the RXR agonist retinoid from an inactive to an active concentration in combination with specific PPAR agonists like, WY14643, troglitazone and cabaprostacyclin, on differentiation and growth in cultured primary sebocytes [72].

2.7.3 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS

Peroxisome proliferator activated receptors (PPAR) belong to the nuclear hormone family. PPAR require heterodimerization with retinoid x receptors to allow their binding to DNA as ligand activated transcription factors. This regulates the expression of target genes encoding peroxisome proliferator's response elements within their promoter regions. PPAR have been shown to regulate multiple lipid metabolic genes in peroxisomes, microsomes and mitochondria by activating PPAR response elements (PPRE) (Figure 9) [48, 140].

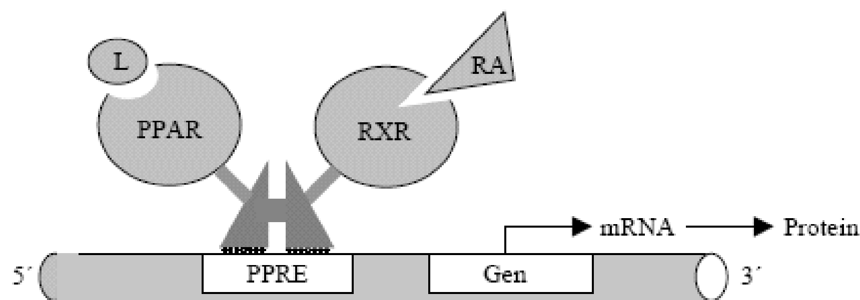


Figure 9 - Scheme for activation of PPAR: Activated PPAR act through PPAR response elements to up regulate multiple genes encoding enzymes involved in peroxisomal, microsomal and mitochondrial β-oxidation, fatty acid ω-hydroxylation, ketone body formation, and fatty acid synthesis as well as fatty acid binding proteins, lipoprotein lipase and apolipoproteins. (L: PPAR ligand; RA: Retinoic Acid = RXR-activating ligand; PPRE: PPAR response element) [98, 129].

All PPAR subtypes are expressed in rat preputial sebocytes, SZ95 and SEB-1 immortalized cell lines and the human sebaceous gland [19, 30, 132, 167]. PCR studies of Chen et al. and Alestas et al. demonstrated that all PPAR subtypes are present in the sebaceous gland and in the pilosebaceous ducts of healthy, acne involved and acne uninvolved skin [4, 19]. Kim showed a significant PPARβ activity in preputial rat sebocytes. PPARα and γ were also activated but to lower levels; however no other study verifies these results. Adult rat sebocytes express mRNA of PPARα, β, $\gamma_{1,}$ but PPARγ_2 mRNA was not detectable [72, 130]. In sebaceous gland cell line SZ95 PPARα, δ, $\gamma_{1,2}$ expression was detectable by RT PCR [19].

Table 6 - Peroxisome proliferator-activated receptor subtypes and their functions

PPAR	Function	Reference
α	• Skin barrier function • Lipid catabolism • Activation of β-oxidation of fatty acids • Regulation of inflammation • Cell differentiation and proliferation • Skin wound healing	[61, 78, 167]
β/δ	• Lipid anabolism • Regulation of late stage sebocyte differentiation • Control of inflammation	[27, 78]
$\gamma_{1,2}$	• Cell apoptosis • Lipid catabolism • Cell proliferation • Skin wound healing • Involvement in oxidative stress	[61, 78, 181]

PPARα, δ, $\gamma_{1,2}$ were initially studied in skin because of their known role in regulating lipid metabolism. PPARα seems to contribute to the skin barrier function, activation of β-oxidation of fatty acids as well as regulation of inflammation, and plays a role in sebocyte differentiation. PPARβ (animals) / δ (human) regulates the late stages of sebaceous cell differentiation and is described as the most effective isotype in stimulating lipid production [27]. PPARγ activates sebocyte development (proliferation) and lipogenesis. PPARγ is also involved in oxidative stress mediated prostaglandin E_2 production and induces COX-2 expression in human SZ95 sebocytes (Table 6) [181]. In SZ95 sebocytes PPARγ is expressed and activated by UVB irradiation. Tert-buthylhydroperoxide (TBH) is a lipid soluble oxidant, which activates PPARγ. After sebocyte treatment with TBH or PPARγ agonist an increased level of COX-2 protein, mRNA expression and prostaglandin E_2 (PGE_2) production were observed. By pre-treatment with PPARγ antagonist GW 9662 the ability of PPARγ and TBH to produce PGE_2 and COX-2 in sebocytes was blocked [181].

Table 7 – Effect of Peroxisome proliferator-activated receptor agonists on lipid synthesis in sebocytes

PPAR	PPAR ligands	Lipid	Rodent sebocytes	Human sebocytes
α	WY14643	↑	[2, 19, 131, 132]	[19]
	LA	↑	[72]	
	GW 0742	↑		[181, 191]
	GW 2433	↑	-	[181, 191]
	GW 7647	↑	-	[181, 191]
	$cPGI_2$	-	[72]	
	AA	↑	-	[4, 191]
	LTB_4	↑	-	[4, 191]
β/δ	GW 0742	↑	-	[181]
	GW 2433	↑	-	[181]
	$cPGI_2$	-	[72, 131, 132]	-
	LA	↑	[72, 131, 132]	[4, 19, 191]
γ	TRO	↑	[2, 63, 72, 131, 132]	-
	BRL	↑	[2, 72, 131, 132]	-
	CIG	-	-	[181]
	GW 4148	-	-	[147, 167]
	Pioglitazone	-	-	[167]
	15-HETE	↑	-	[4]
	Δ15-PGJ2	↑	[63]	-

This table summarizes the activating ligands for all PPAR isotypes. No data (-), linoleic acid (LA), arachidonic acid (AA), leukotriene B_4 (LTB_4), WY14643, ciglitazone (CIG), carbaprostacyclin ($cPGI_2$), troglitazone (TRO), hydroxyeicosatetraenoic acid (15-HETE), rosiglitazone (BRL), pioglitazone, 15-deoxy-delta(^{12,14})-prostaglandin J_2 (Δ15-PGJ_2) synthetic ligands are GW 0742, GW 7647, GW 2433 and GW 4148

If cells express PPAR, they are not necessarily endogenously active; however, they can be activated by ligands depending on the differentiation stage of the cells. PPARα, PPARδ and PPARγ agonists have led to no differences in biological activity between SZ95 and SEB-1 sebocytes [19, 167].

Most natural and synthetic PPAR ligands induce lipogenesis in sebocytes *in vitro* and *in vivo*.

Linoleic acid and carbaprostacyclin ($cPGI_2$) are non-specific neutral PPARα and δ ligands. Arachidonic acid, whose 5-lipoxygenation metabolite leukotriene B_4 is a natural PPARα ligand, was found to induce genes that are associated with β-oxidation. The intermediate metabolite 15-HETE is a PPARγ ligand as well as $cPGJ_2$. Lipid synthesis in human sebocytes is also stimulated by the prostaglandins PGE_2 and 15-deoxy-delta(^{12,14})-prostaglandin J_2 (Δ15-PGJ_2), a neutral PPARγ ligand. PPARγ is down regulated by phytooestrogen and genistein. 17β-Estradiol induces metabolism of PGD_2 to Δ12-PGJ_2.

Sebocytes treated with synthetic PPAR ligands like WY14643, troglitazone or rosiglitazone significantly induced intracellular neutral lipids [2, 63, 162, 168].

However, studies in cultured sebaceous glands led to controversial results. BRL 49653 and WY14643 inhibited sebaceous lipogenesis [30] (Table 7).

Rosenfield tested PPAR activators with and without androgen DHT on preputial rat sebocytes. Growth and maturation of sebocytes are influenced by steroids, testosterone, and the metabolite 5α-dihydrotestosterone (DHT). Rosenfield concluded that the activation of any PPAR seems to increase lipogenesis in the cells. PPARγ seems to be significant in initiating the differentiation of sebocytes. DHT and BRL treatment has an additive effect on differentiation of sebocyte primary culture cells [84, 130].
Zileuton, a potent PPARα antagonist, is an inhibitor of leukotriene B_4 synthesis, which reduces lipid synthesis [194]. The PPARγ antagonist GW 0072 has the ability to inhibit thiazolidinedione induced adipocyte differentiation, but in a study with SEB-1 sebocytes no statistically significant changes in total lipid production were observed [160].

2.8 INFLAMMATORY MECHANISMS IN SEBOCYTES

Inflammation is characterized by the action of active lipid mediators, such as leukotrienes (LT), prostaglandins (PG) and 15-hydroxyeicosatetraenoic acids (15-HETE). These molecules are metabolites from arachidonic acid or linoleic acid processed by the enzymes cyclooxygenase (COX) and lipoxygenase (LOX). Cyclooxygenase, a prostaglandin endoperoxide H synthetase, is a rate limiting enzyme complex for the production of prostaglandins [60, 164]. Cyclooxygenase has two isozymes, COX-1 and COX-2, which are expressed in human sebocytes *in vitro;* moreover, COX-2 expression was selectively up regulated in acne-involved sebaceous glands *in vivo*. COX expression was measured by examining mRNA and protein levels [4]. In a hamster sebocyte study of Iwata et al., COX-2 expression was detected, whereas COX-1 was not.

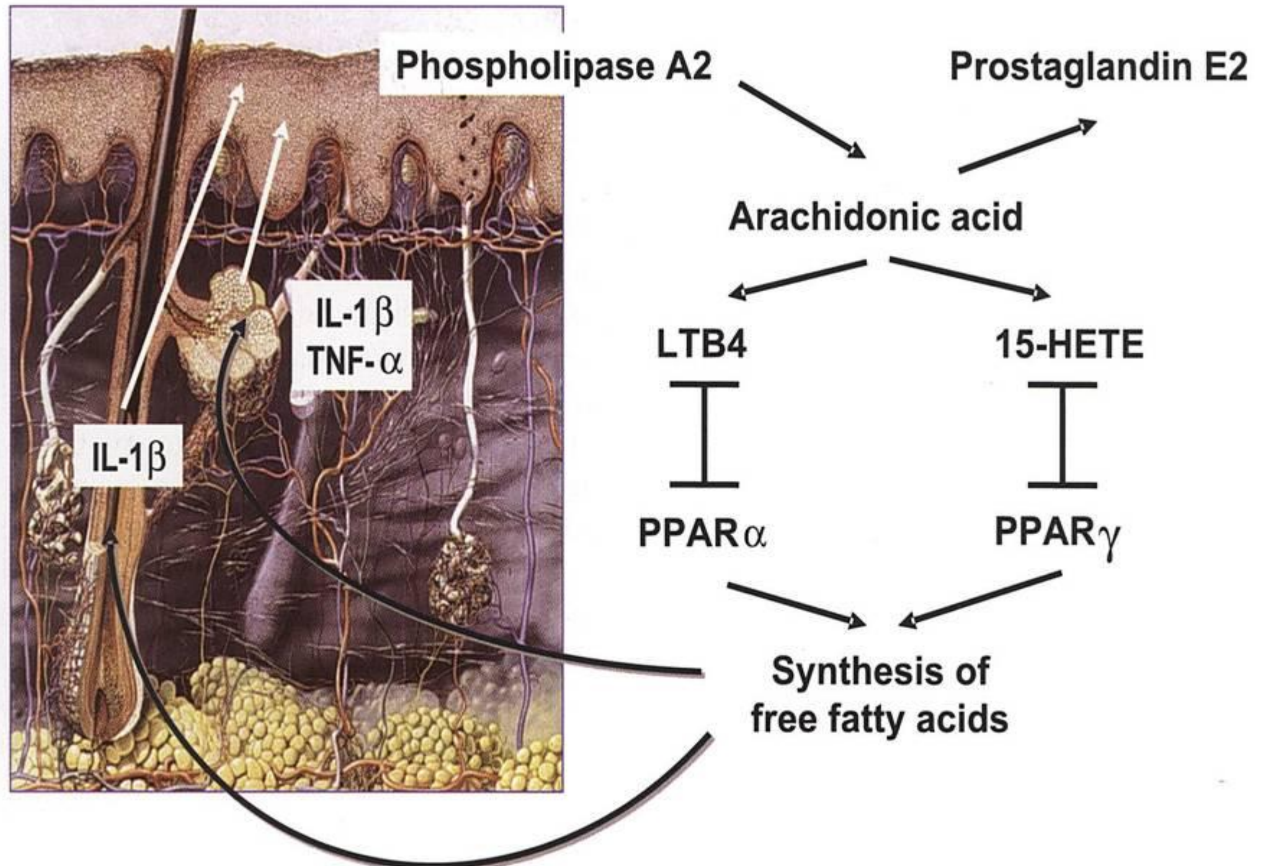


Figure 10 - Arachidonic acid metabolism. Arachidonic acid metabolites are PPAR ligands. PLA_2 activate the arachidonic acid synthesis. The COX-2 is a rate limiting enzyme complex for the production of prostaglandin. The LTB_4 is catalyzed by LOX and LTA_4 hydrolase. All these ligands activate free fatty acid synthesis and cytokine expression in sebocytes [183].

The biological pathway of prostaglandin plays an important role in sebaceous gland cells. Prostaglandin is responsible for the regulation of cellular functions under physiological and pathological conditions.

Prostaglandins (PG) are associated with the development of sebaceous glands and sebocyte lipogenesis. In 1986, prostaglandin synthesis of prostaglandin D_2 (PGD_2) and prostaglandin E_2 (PGE_2) was discovered in mouse sebaceous gland cells [58]. Iwata et al. investigated the involvement of prostaglandins in the formation of intracellular lipid droplets in hamster sebaceous glands. In the augmentation of lipid droplet formation 15-desoxy-delta(^{12,14})-prostaglandin J_2 (Δ15-PGJ_2) is a crucial factor, increasing triglycerol synthesis in sebocytes. COX-2 inhibitors reduce $PGF_{2\alpha}$ and PGE_2 levels but increase Δ15-PGJ_2 production and triacylglycol synthesis. Additionally the synthesis of Δ15-PGJ_2 is mediated by COX-2 and cytochrome P-450 dependent pathways [63]. Zouboulis proposed that for the future treatment of acne Iwata's study may provide insight into the inhibition of the proinflammatory and sebotropic activities. Additionally it shows that a possible regulation of PPARα may be more powerful than the inhibition of the prostaglandin pathway, including PPARγ_1 regulation [188].

The recent study of Zhang et al. demonstrates that the production of PGE_2 is induced by platelet-activating factor receptor (PAF-R) activation and mediated by COX-2 expression. PAF-Rs seem to be involved in regulating the expression of inflammatory mediators, including COX-2, PGE_2, and IL-8 [182].

Cultured SZ95 sebocytes express 5-lipoxygenase (LOX) and LTA_4 hydrolase on the protein and mRNA level. These enzymes are essential for the formation of leukotriene B_4 (LTB_4). When SZ95 sebocytes were treated with arachidonic acid, LTB_4 synthesis was increased. Treatment of human cultured sebocytes with arachidonic acid and calcium ionophore induced expression of 5-lipoxygenase detectable at mRNA and protein level [4, 76]. Immunohistochemical studies revealed weaker staining of 5-LOX and LTA_4 hydrolase in healthy individuals and in uninvolved skin of acne patients than in acne lesions [4].

IL-6 and IL-8 were produced by SZ95 sebocytes *in vitro* and were up regulated in the presence of the 5-LOX activators, arachidonic acid and calcium ionophore [4]. It seems that 5-LOX activation induces cytokine secretion. Squalene peroxide induced the enzymatic activity of 5-LOX in cultured keratinocytes and increased the production of IL-6. Moreover squalene peroxide activated nuclear factor κB (NFκB), which also mediate proinflammatory signaling in cells [110].

Furthermore, sebaceous gland cells can produce cytokines without any mediation of other cells. IL-1α mRNA and protein are detectable in cultured sebocytes at steady state. IL-1β, -6, -8 and TNF-α are released by cultured human SZ95 sebocytes [4]. TNF-α, IL-1α and β are detected at mRNA level by in situ hybridization in normal sebaceous glands [13] and IL-1α by immunohistochemistry in skin biopsies of healthy patients [64].

Neuropeptides may also play an important role in induction of inflammatory processes. Toyoda and Morohashi reviewed the involvement of neuropeptides, neuropeptides degrading enzymes and neurotrophic factors in the inflammatory process of acne [166].

Substance P is associated with multiple cellular responses. It promotes proliferation and differentiation of sebaceous glands *in vitro* and the release of proinflammatory cytokines and chemokines. Toyoda and Morohashi examined the effects of substance P and neutral endopeptidase (NEP) on sebaceous glands in cultured skin. Immunohistochemical studies revealed that germinate sebocytes in acne patients express nerve growth factor [15].

Nuclear factor κB and activator protein-1 are active in acne lesions, leading to increased expression of their target genes such as inflammatory cytokines, enzymes and matrix-degrading metalloproteases .[65].

Sebocytes and keratinocytes in culture secreted matrix metalloproteases (MMP) which have a predominant role in inflammation matrix remodeling and hyperproliferative skin disorders. SZ95 sebocytes secreted pro MMP-2 and pro MMP-9 which was confirmed by microarray analysis [111].

3 AIM OF THE STUDY

The primary aim of this work was to search for extracts and fractions derived from plants, bacteria, *Bothrops moojeni* snake venom and peptides which influence lipid secretion in human sebocytes. If lipid regulating compounds could be discovered, the second goal was to gather more knowledge about these lipid synthesis interactions in SZ95 sebocytes.

In detail, the aim of the study was:

- To establish and optimize a SZ95 sebocyte drug screening model to search for new biologically active ingredients which regulate lipogenesis by measuring neutral lipid production, polar lipid production, cell vitality, and cytotoxicity.
- To test the lipid regulating effects of new compounds on SZ95 sebocytes lipid synthesis three different pre-stimulations with arachidonic acid, linoleic acid and unstimulated should be used.
- To identify lipid stimulating compounds. The active compounds should be discovered, purified and again tested for their lipid regulating activity in a dose-dependent manner in SZ95 sebocytes.
- To exclude apoptotic effects after lipid synthesis stimulation in SZ95 sebocytes. An apoptosis assay could measure caspase-3/7 release in treated SZ95 sebocytes. Only compounds that would not affect viability of the cells should be further investigated.
- To investigate how the lipid stimulating compounds interact in SZ95 sebocytes. The lipid stimulating compounds could activate specific PPARα, β/δ or γ in SZ95 sebocytes transiently expressing PPAR. A new PPAR receptor gene assay for SZ95 sebocytes had to be developed to identify new PPAR ligands. Cells could be transiently transfected with PPARα, δ or γ and treated with compounds to investigate the activation of PPARα, δ and γ.
- To test that treatment of sebocytes with lipid regulating compounds did not cause the expression of inflammatory mediators. Cytokine expression should be evaluated by Luminex 100 system. In addition to that, a cytokine expression profile from SZ95 sebocytes could be created.
- To get more knowledge about the lipid synthesis activation and its pathway, a COX-2 inhibitor, a LOX inhibitor and a PLA_2 inhibitor could be tested for their lipid regulating effects and cytokine expression in arachidonic acid pre-stimulated human SZ95 sebocytes.
- To obtain more knowledge about the specific or unspecific PPAR binding by PPAR ligands e.g. fatty acids. COX-2 and LOX inhibitors could be investigated on arachidonic acid pre-stimulated SZ95 sebocytes transiently expressing PPARα, δ and γ.

The findings should provide a better insight into lipid synthesis and cell signaling mechanism of sebocytes. Additionally, new active compounds and involved pathways should be identified.

4 MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 REAGENTS

Reagent	Distributor
Accutase	Sigma, Munich, Germany
Apoptose Reagent	R&D System, Minneapolis, USA
Arachidonic acid	Sigma, Munich, Germany
Bulk isolute® sorbent Hydromatrix	Separtis GmbH, Grenzach Wyhlen, Germany
Casiton	Scharfe System GmbH, Reutlingen, Germany
Cytosolic PLA_2 Inhibitor ($AACOCF_3$)	US Biological, Massachusetts, USA
DMSO	Fluka, Steinheim, France
Ethanol	Alcosuisse, Delemont, Switzerland
Gentamycin	PAN Biotec GmbH, Aidenbach, Germany
GHK	Bachem, Basel, Switzerland
Hoechst 33342	Invitrogen, Basel, Switzerland
Hydrocortisone	Sigma, Munich, Germany
Linoleic acid	Sigma, Munich, Germany
Lipofectamine 2000	Invitrogen, Basel, Switzerland
MK886	Cayman Chemical, Ann Arbor, USA
NDGA	Sigma, Munich, Germany
Nile Red	Sigma-Aldrich, Steinheim, Germany
NS398	Merck, Darmstadt, Germany
Optimem	Invitrogen, Basel, Switzerland
PBS	PAN Biotec GmbH, Aidenbach, Germany
Phospholipase A_2, snake venom	Sigma-Aldrich, Steinheim, Germany
PLA_2 bee venom	Sigma-Aldrich, Steinheim, Germany
PPARα Ligand (WY14643)	ChemSyn laboratories, Lenexa, USA
PPARβ/δ Ligand (L165041)	Centre for Integrative Genomics, University Lausanne, Switzerland (Prof. Wahli)
PPARγ Ligand (Rosiglitazone)	Alexis Biochemicals, Lausen, Switzerland
Streptavidin-PE	Prozyme, San Leandro, USA
SuperFect® Transfection Reagent	Qiagen, Basel, Switzerland
Triton-X-100	Sigma-Aldrich, Steinheim, Germany
Trypsin	PAN Biotec GmbH, Aidenbach, Germany

4.1.2 TESTED COMPOUNDS

Bothrops moojeni venom (snake venom, Pentapharm, Uberlândia, Minas Gerais, Brazil) was partly purified by HPLC and kindly provided by Anna Maria Perchuc at Pentapharm.

Table 8 - Peptides

Peptides	Distributor
GHK	Bachem, Bubendorf, Switzerland
GHK	Pentapharm, Aesch, Switzerland
TAT-GHK	Pentapharm, Aesch, Switzerland
Cu-GHK	Pentapharm, Aesch, Switzerland
GAA LJ 562-13	Pentapharm, Aesch, Switzerland
GAA LJ 562-16	Pentapharm, Aesch, Switzerland
GAA LJ 562-15	Pentapharm, Aesch, Switzerland
HADGVF-OH 21-004	Pentapharm, Aesch, Switzerland
SVSEIQLMHN 21-003	Pentapharm, Aesch, Switzerland
179-145	Pentapharm, Aesch, Switzerland
179-176 B	Pentapharm, Aesch, Switzerland
GAA LJ 562-14	Pentapharm, Aesch, Switzerland

The used peptides were synthesized by Dr. Heidl, Dr. Fragale and Mr. Wikstroem.

Table 9 - Marine microbial mats

Microbial organism	Distributor
Biolip P 50	Biolip, Tahiti, French Polynesian islands
Biolip P 51	Biolip, Tahiti, French Polynesian islands
Biolip P 75	Biolip, Tahiti, French Polynesian islands
Biolip P 90	Biolip, Tahiti, French Polynesian islands
Biolip P 19	Biolip, Tahiti, French Polynesian islands
Biolip P 2	Biolip, Tahiti, French Polynesian islands
Biolip P 2 supernatant	Biolip, Tahiti, French Polynesian islands
Biolip P 51 supernatant	Biolip, Tahiti, French Polynesian islands

Table 10 - Plants

Family	Plant Name	Part of plant	Distributor
Ranunculaceae	*Actea racemosa*	root	Dixa AG, St. Gallen, Switzerland
Rutaceae	*Aegle marmelos*	root	Treiber, Waibstadt, Germany
Rubiaceae	*Asperula odoratae*	herb	Chrüterhüsli, Basel, Switzerland
Balanitaceae	*Balanites aegyptiaca*	fruit/seed	Treiber, Waibstadt, Germany
Betulaceae	*Betula verrucosa*	leaf	Treiber, Waibstadt, Germany
Brassicaceae	*Brassica oleraceae*	sprout	Ikeda Corporation, Tokyo, Japan
Anacardiaceae	*Buchanania lanzan*	seed	Treiber, Waibstadt, Germany
Fabaceae	*Butea monosperma*	flower	Treiber, Waibstadt, Germany
Compositae	*Calendula officinalis*	flower	Chrüterhüsli, Basel, Switzerland
Fabaceae	*Cassia alata*	herb	Friedrich Nature Discovery, Euskirchen, Germany
Celastraceae	*Cassia glauca*	root	Treiber, Waibstadt, Germany
Apiaceae	*Centella asiatica*	leaf	Chrüterhüsli, Basel, Switzerland
Ranunculaceae	*Cimicifuga racemosa*	rhizome, root	Chrüterhüsli, Basel, Switzerland
Asclepiadaceae	*Marsdenia condurango*	bark	Friedrich Nature Discovery, Euskirchen, Germany
Salicaceae	*Salix purpurea*	bark	Treiber, Waibstadt, Germany
Umbelliferae	*Crithmum maritinum*	herb	Agrimer, Plouguerneau, France
Loranthaceae	*Dendrophtoe pentandra*	leaf	Treiber, Waibstadt, Germany
Dioscoreaceae	*Dioscorea alata*	herb	Friedrich Nature Discovery, Euskirchen, Germany
Dioscoreaceae	*Dioscorea esculenta*	herb	Friedrich Nature Discovery, Euskirchen, Germany
Dioscoreaceae	*Dioscorea persimilis*	herb	Friedrich Nature Discovery, Euskirchen, Germany
Dioscoreaceae	*Dioscorea persimilis*	root	Friedrich Nature Discovery, Euskirchen, Germany
Dioscoreaceae	*Dioscorea tokoro*	herb	Friedrich Nature Discovery, Euskirchen, Germany
Dioscoreaceae	*Dioscorea tokoro*	root	Friedrich Nature Discovery, Euskirchen, Germany
Dioscoreaceae	*Dioscorea villosa*	root	Chrüterhüsli, Basel, Switzerland
Aspidiaceae	*Dryopteris filix-mas syn.*	rhizome	Chrüterhüsli, Basel, Switzerland
Compositae	*Elephantopus scaber*	root	Friedrich Nature Discovery, Euskirchen, Germany
Araliaceae	*Eleutherococcus senticosus*	root	Chrüterhüsli, Basel, Switzerland
Myrsinaceae	*Embelia ribes*	fruit	Treiber, Waibstadt, Germany
Chenopodiaceae	*Fredolia aretioides*	herb	Friedrich Nature Discovery, Euskirchen, Germany
Rubiaceae	*Galium mollugo*	herb	Treiber, Waibstadt, Germany
Ginkgoaceae	*Ginkgo biloba*	leaf	Chrüterhüsli, Basel, Switzerland
Fabaceae	*Glycyrrihza glabra*	stipe	Chrüterhüsli, Basel, Switzerland
Verbenaceae	*Gmelina arborea*	leaf	Treiber, Waibstadt, Germany
Euphorbiaceae	*Jatropha gossypifolia*	root	Dr. Gonzales, Santiago de Cuba,Cuba
Lamiaceae	*Leonurus cardiaca*	leaf	Chrüterhüsli, Basel, Switzerland
Brassicaceae	*Lepidium meyenii*	root	Friedrich Nature Discovery, Euskirchen, Germany
Sapindaceae	*Litchi chinensis*	seed	Ikeda Corporation, Tokyo, Japan
Marsileaceae	*Marsilea quadrifolia*	leaf	Treiber, Waibstadt, Germany
Fabaceae	*Melilotus officinalis*	herb	Friedrich Nature Discovery, Euskirchen, Germany
Labiatae	*Mentha piperita*	leaf	Chrüterhüsli, Basel, Switzerland
Myrothamnaceae	*Myrothamnus flabellifolius*	herb	Friedrich Nature Discovery, Euskirchen, Germany
Nelumbonaceae	*Nelumbo nucifera*	flower	Treiber, Waibstadt, Germany
Cactaceae	*Opuntia ficus indica*	fruit	Friedrich Nature Discovery, Euskirchen, Germany
Aaliaceae	*Panax gingseng*	root	Chrüterhüsli, Basel, Switzerland
Polypodiaceae	*Polypodium vulgare*	rhizome	Chrüterhüsli, Basel, Switzerland
Rosaceae	*Prunus persica*	flower	Friedrich Nature Discovery, Euskirchen, Germany
Rosaceae	*Prunus spinosa*	flower	Friedrich Nature Discovery, Euskirchen, Germany
Punicaceae	*Punica granatum*	fruit	Friedrich Nature Discovery, Euskirchen, Germany
Fagaceae	*Quercus suber*	bark	Friedrich Nature Discovery, Euskirchen, Germany
Rosaceae	*Rhodiola rosea radix.*	root	Floros, Barnaul, Russia
Rosaceae	*Rosa canina L.*	fruit	Chrüterhüsli, Basel, Switzerland
Rubiaceae	*Rubia cordifolia*	root	Treiber, Waibstadt, Germany
Rubiaceae	*Rubia cordifolia*	stipe	Treiber, Waibstadt, Germany
Asparagaceae	*Ruscus aculeatus* L.	rhizome	Chrüterhüsli, Basel, Switzerland
Selaginellaceae	*Selaginellia lepydophylla*	herb	Friedrich Nature Discovery, Euskirchen, Germany
Compositae	*Silybum marianum*	fruit	Friedrich Nature Discovery, Euskirchen, Germany
Smilacaceae	*Smilax sarsaparilla*	root	Chrüterhüsli, Basel, Switzerland
Caryopyllaceae	*Stellaria media*	herb	Treiber, Waibstadt, Germany
Boraginaceae	*Symphytum officinalis*	root	Chrüterhüsli, Basel, Switzerland
Myrataceae	*Syzygium cuminii*	seed	Treiber, Waibstadt, Germany
Compositae	*Taraxacum officinalis*	leaf	Chrüterhüsli, Basel, Switzerland
Combretaceae	*Terminalia arjuna*	bark	Treiber, Waibstadt, Germany
Leguminosae	*Trifolium pratense L.*	flower	Chrüterhüsli, Basel, Switzerland
Graminae	*Trisetum flavescens* L.	seed	Chrüterhüsli, Basel, Switzerland
Tropaeolaceae	*Tropalolum majus*	leaf	Treiber, Waibstadt, Germany
Scrophulariaceae	*Verbascum thapsus*	leaf	Treiber, Waibstadt, Germany
Verbenaceae	*Verbena officinalis*	leaf	Chrüterhüsli, Basel, Switzerland
Violaceae	*Viola tricolor*	flower	Chrüterhüsli, Basel, Switzerland
Verbenaceae	*Vitex agnus castus*	fruit	Chrüterhüsli, Basel, Switzerland
Rhamanaceae	*Ziziphphus lotus*	leaf	Friedrich Nature Discovery, Euskirchen, Germany

4.1.3 PLASMIDS FOR TRANSFECTION

Plasmid	Origin
PPRE-tk Luc	The Salk Institute, La Jolla, USA (Dr. Evans)
PPARα	National Cancer Institute, Bethesda, USA (Dr. Gonzalez)
PPARβ/δ	Centre for Integrative Genomics, University Lausanne, Switzerland (Dr. Perroud)
PPARγ	Centre for Integrative Genomics, University Lausanne, Switzerland (Dr. Perroud / Dr. Michalik)
expression vector pSG5	Promega, Southampton, United Kingdom

Plasmid	Depiction of the sequence of the C-terminal ends
PPARα	LLNVKPIEDIIQDNLLQALELQLKLNHPESSQLFAK VLQKMTDLRQIVTEHVQLLHVIKKTETDMSLHPLL QEIYKDLY$_{505}$
PPARβ/δ	LMNVPQVEAIQDTILRALEFHLQVNHPDSQYLFP KLLQKMADLRQLVTEHAQMMQWLKKTESETLLH PLLQEIYKDMY$_{440}$
PPARγ	LLNIGYIEKLQEGIVEVLKLHLQSNHPDDTFLFPK LLQKMVDLRQLVTEHAQLVIKKTESDAALHPLLQ EIYRDMY$_{468}$

4.1.4 CELL CULTURE MATERIALS

Material	Distributor
12-well plates	Nunc, Wiesbaden, Germany
96-well plates	Nunc, Wiesbaden, Germany
Cell culture flasks	Nunc, Wiesbaden, Germany
Centrifuge tubes	Nunc, Wiesbaden, Germany
Cover slip	Menzel-Glaser, Braunschweig, Germany
Culture slides Camber 6-8	BD Falcon, Becton Dickinson, USA
Freezing vials	Nunc, Wiesbaden, Germany
Microscope slide	Menzel-Glaser, Braunschweig, Germany
MultiScreen® Solubility Filter Plate	Millipore, Billerica, USA
Serological Pipette	Sarstedt, Nümbrecht, Germany
Sterile filters (0.2 μm)	Corning, Acton, USA
Tube	Sarstedt, Nümbrecht, Germany

4.1.5 ACCESSORIES

Accessories	Distributor
Teflon silicone septa	Chase Scientific Glass, Tennessee, USA
Filter	Dionex GmbH, Idstein, Germany
Fluoro Filter 544 nm	Catalys AG, Wallisellen, Switzerland
Fluoro Filter 555 nm	Catalys AG, Wallisellen, Switzerland
Fluoro Filter 620 nm	Catalys AG, Wallisellen, Switzerland

4.1.6 BUFFERS

Buffer	Distributor
Reporter lysis buffer	Promega, Madison, USA
Passive lysis buffer	Promega, Madison, USA
Luminex buffer	Invitrogen, Basel, Switzerland
Bio Rad buffer	Bio Rad Laboratories, Hercules, USA

4.1.7 CELLS

Cells	Origin
SZ95 sebocyte cell line	Dermato-endocrinology, Charité Berlin, Germany (Prof. Zouboulis)
HeLa cell line	Centre for Integrative Genomics, University Lausanne, Switzerland (Prof. Wahli)
COS/7 cell line	Centre for Integrative Genomics, University Lausanne, Switzerland (Prof. Wahli)

"The SZ95 sebaceous gland cell line refers to a cell line resulting from the transfection of human sebaceous gland cells derived from isolated facial sebaceous glands with a PBR-322 based plasmid containing the coding region for the simian virus 40 large T gene. The cell line has been immortalized by Prof. Dr. Christos C. Zouboulis. Prof. Zouboulis owns the relevant patents EP1151082, DE59913210D, AT319813T, AU770518B, CN1344314T, DK1151082T, KR689120, PL194865 and has filed further patent applications in several countries (CA2360762, HU0200048, IL144683D, JP2002535984T, US2002034820 and others), which claim the cell line and uses thereof."

All cells (SZ95, HeLa and COS/7) were maintained at 37°C, 5% CO_2.

4.1.8 CELL CULTURE MEDIA AND SOLUTIONS

Sebocyte HSG-Medium	Distributor
Sebomed® Basal Medium 500 ml	Biochrom, Berlin, Germany
10% fetal calf serum	PAN Biotec GmbH, Aidenbach, Germany
50 µg/ml gentamycin	PAN Biotec GmbH, Aidenbach, Germany
1 mmol $CaCl_2$	Sigma, Munich, Germany
5 ng/ml epidermal growth factor (EGF)	Sigma, Munich, Germany

Sebocyte Medium for Transfection	Distributor
Sebomed® Basal Medium 500ml	Biochrom, Berlin, Germany
10% fetal calf serum without lipids	PAN Biotec GmbH, Aidenbach, Germany
50 µg/ml gentamycin	PAN Biotec GmbH, Aidenbach, Germany
5 ng/ml epidermal growth factor (EGF)	Sigma, Munich, Germany

Medium for COS/7 and HeLa cells	Distributor
DMEM 500 ml	Sigma-Aldrich, Steinheim, Germany
10% fetal calf serum	PAN Biotec GmbH, Aidenbach, Germany

Medium for COS/7 and HeLa cells for Transfection	Distributor
DMEM 500 ml	Sigma-Aldrich, Steinheim, Germany

4.1.9 MATERIALS OF CELL CULTURE TECHNIQUE

Proliferation analysis	Distributor
Methyl-umbelliferyl-heptanoate	Sigma, Munich, Germany

Detection of cell lipids	Distributor
Nile Red	Sigma, Munich, Germany

Detection cell vitality	Distributor
FDA	Sigma, Munich, Germany

Cytotoxicity detection Kit-Assay	Distributor
LDH	Roche Diagnostics, Mannheim, Germany

Human Cytokine and Chemocine Assay: Multi-Plex System	Distributor
Bio-Plex human IL-1β Assay	Bio Rad Laboratories, Hercules, USA
Bio Plex human IL-6 Assay	Bio Rad Laboratories, Hercules, USA
Bio Plex human IL-8 Assay	Bio Rad Laboratories, Hercules, USA
Bio Plex human IL12 Assay	Bio Rad Laboratories, Hercules, USA
Bio Plex human IL13 Assay	Bio Rad Laboratories, Hercules, USA
Bio Plex human 8 Plex A Panel	Bio Rad Laboratories, Hercules, USA
Bio Plex Reaction Kit	Bio Rad Laboratories, Hercules, USA

Apoptosis - Caspase-3/7 Assay	Distributor
Apo ONE™ Homogeneous	Promega, Medison, USA
Color Reagent A and Color Reagent B R&D System Cat DY999)	R&D System, Minneapolis, USA

Diverse Kits	Distributor
High speed Purification Kit	Qiagen AG, Hilden, Switzerland
Dual luciferase® reporter assay system	Promega, Medison, USA
PLA_2 activity assay	Assay Designs, Ann Arbor, USA
Immunometric assay for $sPLA_2$-IIA	Cayman Chemical, Ann Arbor, USA

4.1.10 EQUIPMENT / INSTRUMENTS

Instruments	Distributor
Cellcounter Casy1	Scharfe System GmbH, Reutlingen, Germany
Centrifuge 5810R	Eppendorf, Basel, Switzerland
CO_2 incubator AUTO-ZERO	Heraeus, Hanau, Germany
Extractor (ASE100)	DIONEX, Olten, Switzerland
Fluorescence microscope BX51	Olympus, Tokyo, Japan
Fluoroscan Ascent FL	Thermo Labsystems, Helsinki, Finland
Laminar flow (Skan VSE 2000-120)	Skan AG, Basel, Switzerland
Laminar flow (Gelaire BSB6A)	Skan AG, Basel, Switzerland
Libra	Mettler Toledo, Greifensee, Switzerland
Luminex 100 system	Luminex Corporation, Austin, USA
Lumat, LB9501	Berthold, Regensdorf, Switzerland
Chameleon Multilable Detection Pl.	Hidex, Oy, Finland
Lyophilizer (GT2)	Lyovac, Rotkreuz, Switzerland
Microplate Washer	ASYS Hitech GmbH, Eugendorf, Austria
Microscope camera (DP12)	Olympus, Tokyo, Japan
Microscope camera (DP70)	Olympus, Tokyo, Japan
Millipore Multi Screen	Millipore, Billerica, USA
Mixer	Telion AG, Schlieren, Switzerland
Mulitidrop 384	Thermo Electron Cooperation, Vantaa, Finland
Multiscan Ascent	Thermo Labsystems, Helsinki, Finland
Nanodrop ND Spectrometer	Witec AG, Fanghöfli Littau, Switzerland
Phase contrast microscope	Olympus, Tokyo, Japan
Pipettes	Eppendorf, Hamburg, Germany
Rotavapor R-134	Büchli, Flawil, Switzerland
Titramax 100	Heidolph Elektro GmbH&Co.KG, Kelheim, Germany
Vacusan R	Scan AG, Basel, Switzerland
Vortexer	Bender&Hobein AG, Zürich, Switzerland

4.2 METHODS

4.2.1 SELECTION OF COMPOUNDS

Natural products provide a greater structural diversity than standard combinational chemistry and thus offer major opportunities for finding novel low molecular weight structures that are active against a wide range of assay targets. This study focused on the discovery of natural products acting on lipogenesis or lipolysis.
For this study, plants, microbial organism samples and *Bothrops moojeni* snake venom were selected. Most of the plants were chosen by literature search. The criterion for the search was wound healing, skin lipid regulation, and hormonal regulation.
The microbial organisms were from the French Polynesian atoll. Until now, bioactivity and biodiversity of these samples were poorly investigated. However, these organisms might be a rich source for new drugs. These substances have to be identified for their pharmaceutical activity. The venom of the South American lance-head viper, *Bothrops moojeni* may induce a wide spectrum of pharmaceutical effects. Peptides like GHK were selected by literature research (wound healing). Other peptides were developed at Pentapharm.

4.2.1.1 EXTRACTION OF PLANT MATERIAL

Numerous medicinal plants are used for disease treatment. Often not only one compound but a variety of them is responsible for pharmaceutical effects (of medicinal plants). Only in few cases the full metabolite profile of these extracts and the contributions of individual constituents to the overall pharmaceutical properties are known.

Preparation procedure:

A variety of plant herbs, fruits, barks, seeds, roots and rhizomes were collected. Dried leaves, roots, bark, flowers and seeds were ground. The blended sample, a mixture of 10 g pulverized plant samples and 5 g hydromatrix was put into an accelerated solvent extractor cell and extracted with polar (water) or non polar (ethanol) solvents in a Dionex ASE extractor.

Table 11 - Extraction methods process

Method 1: Solvent: Water	Method 2: Solvent: Ethanol
Temperature: 129°C Static time 5 minutes Flush volume: 60% Purge time 60 seconds Static cycles: 4	Temperature: 90°C Static time 5 minutes Flush volume: 60% Purge time 60 seconds Static cycles: 4

It is known that ethanol has a proliferative effect on SZ95 sebocytes. For this reason the extracts were evaporated to dryness with a rotary evaporator at 40°C and 70 bar. Afterwards extracts were dissolved in water. After freezing all extracts a lyophilisation system that removes water and other solvents from frozen samples by sublimation was used.

For profiling studies, dried ethanol extracts were dissolved in DMSO and water extracts in water, to a concentration of 10 mg/ml. These extracts were filtered through 0.2 μm filters for sterile cell culture conditions. All extracts were stored at 4°C.

4.2.1.2 EXTRACTION OF MICROBIAL ORGANISMS

Microbial mats from Polynesian islands are laminated communities, basically composed of phototropic and chemotropic prokaryotes. The vertical stratification of the community is a response of the organisms to the gradients of light, oxygen, sulfide and pH, according to their physiological requirements. Nitrogen fixation is essential for the development of many microbial mats. Mats from the French Polynesian atoll are characterized by a diversity in terms of physical and chemical parameters with pH ranging from 6 to 10.5, salinity ranging from 9 g/l to 42 g/l, temperature ranging from 42°C down to 20°C during the night and finally varying light intensity [7].

The used microbial organism samples contained bacteria like *Pseudoalteromonas*, *Flavobacterium*, *Alteromonas*, *Paracoccus* and *Vibrio* cyanobacteria. These organisms produce exopolysaccharides and pigments.

Preparation procedure:

Microbial samples consist of different bacterial pigments recovered from bacteria isolated from Polynesian microbial mats and their supernatant. Ten milligrams per milliliter of the lyophilized microorganisms were diluted in water. With the help of a pulsed ultrasonic device the cell membranes were disrupted to release the molecules such as bacterial pigments, bioactive molecules and polysaccharides. For cell culture conditions the samples were filtered through a 0.2 μm filter wash.

4.2.1.3 SYNTHESIS AND PREPARATION OF PEPTIDES

Used peptides were synthesized at Pentapharm by Dr. Fragale, Dr. Heidl and Mr. Wikstroem.

4.2.1.4 *BOTHROPS MOOJENI* - PROTEIN SEPARATION

Bothrops moojeni (Botmo) crude venom was collected, pooled and dried at Pentapharm Brazil, Uberlândia. It was transported and stored in desiccated form until

it was reconstituted in deionized water at Pentapharm, Switzerland. A 30% solution was obtained and stored at -80°C (cf. 4.1.2; page 27).

Size exclusion chromatography

Crude venom from *Bothrops moojeni* was fractionated by gel filtration and HPLC. Crude, reconstituted venom was separated into 18 gel filtration (GF) fractions on two in-line Superdex-75 (Pharmacia) XK 26 columns. For the protein elution 50 mM CH_3COONH_4, 150 mM NaCl, pH 7.5 buffer at the flow rate of 2.2 ml/min was used and the absorbance was monitored at λ = 280 nm. Aliquots of 300 mg were loaded onto the system resulting in a good and reproducible separation. To ensure good reproducibility of the process, the columns were regenerated every 5 runs using 0.5 M NaOH (flow rate 1 ml/min, over the period of 8 h). Collected fractions were called Botmo GF 1 - 18, stored at -80°C and used for further separation steps and various measurements [115].

RP-HPLC Botmo gel filtration fractions 11, 12 and 15 (about 10 mg of protein) were then submitted to Solid Phase Extraction by acidification with 0.1% TFA (trifluoroacetic acid) and loaded on Sep-Pak C18 classic cartridges (Waters™). Solvation, equilibrium, sample application and elution were performed according to the manufacturer´s instructions. The fractions eluted with 60% acetonitrile were enriched in peptides and proteins. They were freeze-dried under vacuum in a SC210A Speed Vac™ Plus from Savant™. The desalted fractions were processed by RP-HPLC on a Waters Alliance 2690 System using a semi preparative HPLC column (Vydac #218TP510 protein & peptide C_{18}, 10 mm x 250 mm). Freeze-dried fractions were reconstituted in 0.1% TFA and centrifuged at 11,000 rpm before injections on column. Proteins and peptides were eluted using the gradient of 2-50% of 90% acetonitrile in 0.1% TFA for 75 min, then a gradient of 50 - 60% for 2 min and a gradient of 60 - 80% for the following 3 min, at a flow rate of 3 ml/min. The absorbance was monitored at λ = 225 nm and the fractions were collected manually, following peak absorbance.

4.2.1.5 MEASUREMENT OF PHOSPHOLIPASE ACTIVITY

Secretory phospholipases A (sPLA) is a group of enzymes that upon activation, cleave an acyl ester bond in the sn-2 position in glycerophospholipid. PLA_2 generate arachidonic acid and eicosanoids from membrane phospholipids. For the determination of PLA_2 activity a $sPLA_2$ kit (Assay Designs) was used.

Preparation procedure:

Standards and samples (50 μl) were pipeted in duplicate wells. Reaction buffer (100 μl) and substrate (50 μl) were added and the plate was incubated at 37°C for 30 min. The specific substrates for PLA_2 were converted into a sulfhydryl molecule, which was detected colorimetrically using 25 μl of Ellman´s reagent which formed a yellow colored product with the sulfhydryl molecule.
The amount of $sPLA_2$ in the sample was compared to the amount of standard $sPLA_2$ supplied in the kit by comparison of the yellow color generated at 405 nm measured with a multiscan system.

4.2.1.6 IMMUNOMETRIC ASSAY FOR SECRETED PHOSPHOLIPASE A_2 IIA

Secretory PLA_2 type IA, IIA and IIB are found in venom of snakes like vipers, cobras and rattlesnakes. Since 1994 the $sPLA_2$ IIA are suggested to trigger the release of arachidonic acid in inflammatory processes [23].

Preparation procedure:

This immunometric assay is based on a doublet antibody immune sandwich technique which is specific for PLA_2 IIA. The immunometric assay was used according to the manufacturer's instructions. In brief, each well on a 96-well plate is coated with a monoclonal antibody specific against PLA_2 IIA. Samples and standards (100 µl) were pipeted in duplicate wells. The immobilized antibody binds to any PLA_2 IIA introduced to the well. Then 100 µl of acetylcholinesterase PLA_2 Fab`conjugate was added to all wells except the blank and incubated overnight in the dark at 4°C. Acetylcholinesterase $PLA_2$ Fab`conjugate binds selectivity on the other side of the PLA_2 molecule, a sandwich layout is established. After washing five times to remove the unbound conjugates, 200 µl of Ellman´s reagent was added and incubated for 30 min. This reagent consists of acetylthiocholine and 5,5`-dithio-bis-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by acetylcholinesterase reacts to thiocholine. The non enzymatic reaction of thiocholine with 5,5`-dithio-bis-(2-nitrobenzoic acid produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm. The amount of $sPLA_2$ IIA in the sample was compared to the amount of standard $sPLA_2$ IIA supplied in the kit by comparison of the yellow color generated at 405 - 420 nm measured at multiscan plate reader.

4.2.2 CELL LINE – IMMORTALIZED HUMAN SZ95 SEBOCYTES

Human sebocytes are cells of the sebaceous gland. Normal human sebocytes can only be grown for 3 to 6 passages. To overcome this problem, Zouboulis and colleagues cultured human facial sebocytes from a 87-year-old woman and transfected them with the Simian Virus-40 large T antigen [199].
Immortalized human sebocyte (SZ95) differentiation was characteristic for the intracellular accumulation of neutral lipids in cytoplasm.
Cells used for experiments in this work were between passages 23 and 33. Work with the low passage cell stock was conducted in order to avoid selection of the cells, which may occur at high passage levels.

Preparation procedure:

Culture conditions

SZ95 sebocytes were cultured in 175 square centimeters flasks and detached using trypsin as soon as possible by a standard procedure: The cells were washed twice with PBS without Ca^{2+} and Mg^{2+}. Then fresh trypsin (3 ml) was added and incubated for 20 min at 37°C, 5% CO_2. After cell detachment the reaction was stopped with approximately 6ml of HSG-medium containing fetal calf serum. The cells were pelleted by centrifugation (200 - 300g for 10 min) and then resuspended in HSG

medium. Then 3 million SZ95 sebocytes were transferred into a new flask. Sebocytes reached 90% of confluence after approximately one week.

Thawing cells

The frozen cells were thawed in a water bath at 37°C. The 1 ml cell suspension included in the delivered cryotube was diluted with HSG-medium in order to quickly decrease the concentration of DMSO included in the freezing medium to less than 0.5%. Cell viability was assessed at this point; about 15 - 20% dead cells were present. The SZ95 sebocytes were placed in 175 square centimeters flasks (approximately 1.5 - 3 million cells per flask). The added medium was 0.2 - 0.5 ml medium per cm^2 of growth surface. The medium was changed every second day. Confluency was reached after approximately one week.

Freezing cells

SZ95 sebocytes were detached for freezing at 70 - 80% of confluency using trypsin and resuspended in 1 ml Sebomed culture medium containing 10% DMSO. The cells, approximately 2 - 3 million per 1 ml, were frozen in a cryotube with a freezing speed of 1°C/min.

4.2.3 SCREENING MODEL TO INVESTIGATE SZ95 SEBOCYTE LIPID SYNTHESIS

Established treatments for sebaceous gland diseases act either on lipid production, hyperproliferation, inflammation, differentiation or a combination of these aspects. Depending of the research target the sebocyte culture design varies to investigate sebaceous gland functions, like lipogenesis. This cell culture model was developed to investigate the regulation of lipid synthesis. The screening model was used to search for new active compounds to inhibit or stimulate neutral lipid production in human sebocytes.
Potential new drugs for treatment of acne or dry skin disorders can be identified by this newly developed screening model. Tests were carried out in three 96-well culture plates for each experiment, including Nile Red for staining neutral and polar lipids and FDA staining as viability markers for sebocytes. It was possible to determine multiple parameters from a single well.
In this model a number of compounds were used in a pharmacological concentration range from 10^{-5} to 10^{-4} M, including known anti-acne and lipid stimulating drugs, and unknown compounds in 1/100 or 1/1000 dilutions that were potentially useful in the treatment of acne and dry skin.

Table 12 - Compound concentrations

Compounds	Diluents/Concentration
Water Extracts	100-fold dilution
DMSO Extracts	1000-fold dilution
Hormones	10^{-8} M
Fatty acids	10^{-5} to 10^{-4} M

Preparation procedure:

40,000 cells/well were seeded in 96-well plates and cultured for 24 h. After 24 h cells reached confluency and were pre-treated with 10^{-5} M arachidonic acid, 10^{-4} M linoleic acid or 50 µl/well HSG medium. One hour later the treatment with negative control, hydrocortisone 10^{-4} M, and control medium containing 0.1% solvent (water or DMSO) started. Lipid synthesis in SZ95 sebocytes was decreased by hydrocortisone by about 10 - 20% and increased by fatty acids like arachidonic acid and linoleic acid 0.1 mM by 40%. Compounds for the screening were prepared in deep well plates. The test substances were diluted in 1/100 and 1/1000 dilution steps.
All compounds (pre-treatment and treatment) were cultured for 24 h at 37°C, 5% CO_2.
On the day of evaluation the medium was removed, and cells were washed twice with PBS and treated with Nile Red to stain intracellular polar and neutral cell lipids and FDA for cell vitality detection. After 15 min incubation at 37°C fluorescence was measured as described in chapters 4.2.5 and 4.2.6.

4.2.4 FLUORESCENT LABELING OF MOLECULES AND ORGANELLES IN LIVING CELLS

Fluorescence microscopy allows visualization of specific molecules that emit light when excited by light of a defined wavelength. For fluorescence microscopy, cells are stained with a dye and the dye is illuminated with filtered light at the absorbing wavelength; the light emitted from the dye is viewed through a filter that allows only the emitted wavelength to be seen.
Fluorescence microscopy of living cells is a powerful tool to investigate labeled organelles or structure in living cells. The technique is used to study specimens, which can be made to fluoresce. The fluorescence microscope contains special filters, like DAPI, Texas Red and FITC. The filters are designed to isolate and manipulate two distinct sets of excitation and fluorescence wavelengths.

Table 13 - Visualizing cell structures

Filter	Dye	Cell structure	Wavelength Ex/Em
Texas red	Nile Red	PL	544 / 620 nm
DAPI	Hoechst 33342	DNA	385 / 420 nm
FITC	Nile Red	NL	485 / 555 nm

Preparation procedure: Labeling of cells:

Cells (20,000 cells/chamber) were seeded in chamber slides and cultured for 24 h. Wells were washed with PBS and treated with compounds for 24 h. Then, cells were cultured for another 48 h at 37°C and 5% humidity in the CO_2 incubator.
On the day of evaluation the medium was removed. Cells were washed twice with PBS and treated with a mixture of Nile Red 0.1mg/ml and Hoechst 33342 5 μg/ml.
Stock solution of Hoechst 33342 (10 μg/ml) was prepared in distilled water, protected from light, and stored at 4°C; a 1000-fold dilution in PBS was prepared to be used for labeling.
The Hoechst dye labels the nucleus DNA and emits light of the wavelength λ = 385/420 nm. Nile Red stained intracellular polar and neutral cell lipids for detection. The chamber slides were incubated in the labeling solution at 37°C, 5% CO_2 for 15 -30 min.
Before measuring the labeling medium was aspirated and cells were rinsed three times in PBS. Images were recorded in grey scale with a digital camera coupled to an Olympus fluorescence microscope equipped with band pass emission fluorescence filter optical blocks.

4.2.5 NILE RED STAINING FOR CELL LIPID DETECTION

The development of cytoplasmic lipid droplets is a normal cellular process in cells.
The dye Nile Red (9-diethylamino-5 H-benzo[α]phenoxazine-5-one) (Figure 11**)** can be applied as a fluorescent vital stain for the detection of intracellular lipid droplets by fluorescence microscopy [49, 50] (see also chapter 4.2.4).
The production of lipids is assessed by the Nile Red fluorescence assay. This assay is based on the measurement of fluorescence by a fluorospectrometer after staining the cells with Nile Red, a dual emission lipid dye [198]. Fluorescence is a member of the ubiquitous luminescence processes, in which susceptible molecules emit light from electronically excited status by either a physical (for example, absorption of light), mechanical or chemical mechanism. After staining with Nile Red, the cells were viewed for gold-yellow neutral lipid fluorescence and for red polar lipid fluorescence.

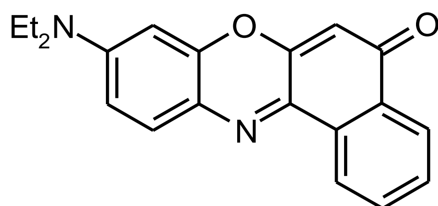


Figure 11 - Structure of Nile Red ($C_{20}H_{18}N_2O_2$)

Preparation procedure:

Sebocytes (40,000 cells/well) were cultured in 96-well plates for 24 h. Then, medium was removed and the cells were treated for 24 h with compounds. After treatment the medium was removed, the wells were washed twice with PBS, and 100 μl of a 10 μg/ml Nile Red solution in PBS was added to each well. The plates were then incubated at 37°C for 15 min, and the released fluorescence was read on a

fluorospectrometer. The results were measured in absolute fluorescence units in comparison with the controls, using 485 nm excitation and 565 nm emission filters for neutral lipids and 540 nm excitation and 620 nm emission filters for polar lipids. Experiments were performed in triplicate with 10 wells evaluated for each data point in each experiment.

4.2.6 FLUORESCEIN DIACETATE VITALITY MEASUREMENT

Fluorescein diacetate (FDA) stains viable cells by uptake into these cells. In cells FDA is hydrolyzed by unspecific esterases which separate FDA in fluorescein and acetate. The dye is accumulated in viable and esterase active cells. Contrary to FDA, fluorescein is not able to pass the intact cell membrane and is stored in vital cells. A green fluorescence of the cell cytosol appears under the fluorescence microscope.

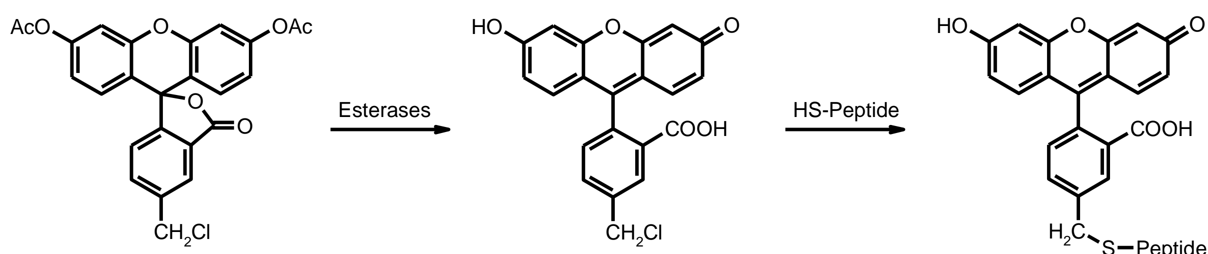


Figure 12 - Principle of fluorescein measurement.

Preparation procedure:

Cells (40,000 cells/well) were seeded in 96-well plates and cultured for 24 h. Wells were washed three times with PBS and afterwards treated with compounds for 24 h. Fluoresceindiacetate powder was diluted in DMSO (1 mg/ml).
SZ95 sebocyte cell suspensions were treated and incubated at 37°C for 15 min with FDA (1 µl/ml). The results were presented in absolute fluorescence units in comparison with the controls. The green fluorescence was measured using 485 nm excitation and 538 nm emission filters by fluorospectrometer. Experiments were performed in triplicate for each data point in each experiment.

4.2.7 DETECTION OF CELL CYTOTOXICITY WITH LACTATE DEHYDROGENASE RELEASE ASSAY

Dying cells release their cell content into the medium. The cytotoxicity detection is based on the measurement of lactate dehydrogenase (LDH) activity which is released from the cytosol of damaged cells into the supernatant.
LDH catalyses oxidation at lactate to pyruvate *in vivo*. At the same time the reduction of NAD^+ to NADH 1H occurs. The enzyme diaphorase and tetrazolium salt 2-[4 - indophenyl]-3[4nitropheny]-5-phenyltetrazolium chloride (INT) are added. Diaphorase catalyzes reduction of INT to red formazan salt by simultaneous oxidation of NADH and H to NAD^+ (Figure 13).

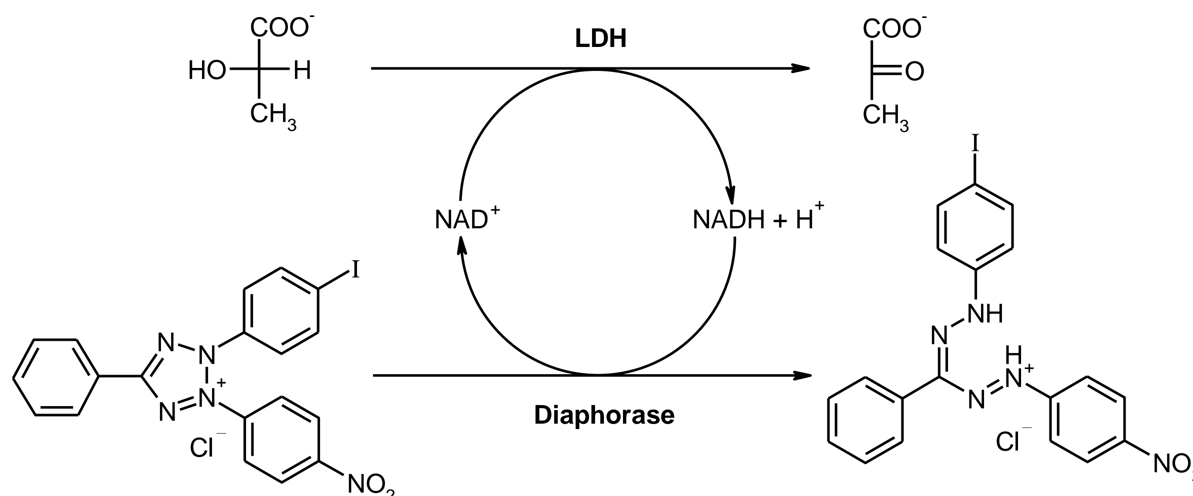


Figure 13 - Principle of lactate dehydrogenase activity. Lactate dehydrogenase (LDH) catalyzed reaction is followed by diaphorase catalyzed reaction.

Preparation procedure:

The LDH kit from Roche Diagnostics was used according to the manufacturer's instructions. In brief, cells (10 000 cells/well) were seeded in 96-well culture plates and cultured for 24 h. Wells were washed with PBS and treated for 24 h with compounds. After 4 - 24 h incubation cell microplates were centrifuged for 10 min at 250 xg and 15 - 25°C. Subsequently cell free culture supernatant (100 µl/well) was transferred to the plates. A reaction mixture of diaphorase, NAD^+, INT and sodium lactate was added to the supernatant and incubated for approximately 15 to 30 min at 25°C protected from light. The resulting red formazan salt was quantified photometrically. Absorbance was measured at 490 nm (reference wavelength 690 nm).
The amount of accumulated formazan was proportional to LDH-activity in supernatant and correlated to number of dead cells. Cytotoxicity effects were clarified as significant if the increase of LDH was over 10% compared to control.

4.2.8 APOPTOSIS ASSAY

Apoptosis is defined as the controlled self destruction program of cells. The apoptotic modes of cell death are active and defined processes. Apoptosis is controlled by several factors like PLA_2 pathway [151, 158] and activation of the caspase family [32, 153].

In humans eleven caspases have been identified. There are two types of caspases: initiator caspases and effector caspases. Initiator caspases (e.g., caspase-8, caspase-9) cleave inactive pro-forms of effector caspases, thereby activating them; effector caspases (e.g., caspase-3, caspase-7) in turn cleave other protein substrates within the cell initiating in the apoptotic process. The initiation of this cascade reaction is regulated by caspase inhibitors. Caspase-3 and -7 play key effector roles in apoptosis in mammalian cells.

Preparation procedure:

Cells (40,000 cells/well) were seeded in plates and cultured for 24 h. The wells were then washed three times with PBS and treated for 24 h with compounds.

The apoptosis assay included a profluorescent caspase-3/7 consensus substrate, rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide) (Z-DEVD-R110), and an optimized bifunctional cell lysis/activity buffer. The buffer efficiently lysed cultured mammalian cells and supported optimal caspase-3/7 enzymatic activity. The substrate and buffer were combined to make the caspase-3/7 reagent. Homogeneous caspase-3/7 reagent (100 µl) was added to each well of a non transparent 96-well plate containing 100 µl of blank, control cells in culture.
Upon cleavage on the C-terminal side of the aspartate residue in the DEVD peptide substrate sequence by caspase-3/7 enzymes, rhodamine 110 becomes fluorescent when excited at a wavelength of 498 nm. The emission maximum was 521 nm. The amount of fluorescent product generated was representative for the amount of active caspase-3/7 present in the sample. Fluorescence was measured every 30 min for 18 h with a fluorescence plate reader.

4.2.9 CYTOKINE ASSAY USING A BIO-PLEX SUSPENSION ARRAY SYSTEM

Cytokines are a group of proteinaceous signaling compounds that, like hormones and neurotransmitters, are used extensively for intercellular communication.
They are critical to the functioning of both innate and adaptive immune responses. Apart from their importance in the development and functioning of the immune system, cytokines play a major role in a variety of immunological, inflammatory and infectious diseases.
Cytokines are produced by a wide variety of cell types and can have effects on both nearby cells or throughout the organism, sometimes strongly dependent on the presence of other chemicals and cytokines. The cytokine family consists mainly of small water-soluble proteins and glycoproteins (proteins with an added sugar chain) with a molecular mass of 8 - 30 kDa.
The multiplex bead array technology provided the means to measure multiple assays simultaneously. It allows the detection of soluble cytokines and chemokines in cell supernatant by capturing these to spectrally district beads and tagging the desired element with fluorescent-labeled markers for analysis.
Multiplexed sandwich immunoassays for cytokines were used by coupling cytokine specific capture antibodies to beads with different emission spectra. The binding of biotinylated detection antibodies bound with a streptavidin-conjugated fluorochrome was analyzed [57].

Preparation procedure:

Sebocytes (40,000 cells/well) were seeded in 96-well microplates and cultured for 24 h. Then cells were treated for 24 h with compounds.

After overnight sebocyte incubation with *Bothrops moojeni* GF11, GHK, HC and Biolip 51 supernatant, 100 µl of cell free supernatants were transferred into a new plate.
In this study SZ95 sebocyte supernatant was investigated for IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, GM-CSF, IFN-γ and TNF-α. Cytokine detection kits from Bio Rad Laboratories were used. The assay was performed in 96-well filter plates using all assay compounds provided in the kit.
Standard curves were generated by using reference concentrations supplied by the manufacturer. The cytokine standard was reconstituted and diluted in the same medium in which the cells were prepared.
The filter plate was pre-wetted with 70 µl of Luminex buffer. The buffer was removed by vacuum filtration on a Millipore Multi Screen. Then 50 µl of the prepared bead working solution was added to the respective wells, sucked dry by vacuum filtration and washed again with Luminex buffer.
Samples were diluted in appropriate HSG medium (2-fold). Diluted standards and samples (50 µl/well) were added to the respective wells and incubated in the dark for 30 min on a shaker at 300 rpm at room temperature. Subsequently the microtiter filter plate was washed again three times with Luminex buffer (70 µl). Buffer was removed by the Millipore Multi Screen system. The detection antibody was prepared to a 1x concentration. Detection antibody (25 µl) was added to the respective wells and incubated in the dark at room temperature for 30 min on the shaker.
Thereafter the wells were washed using the vacuum manifold and detector antibody conjugated to biotin was added. Streptavidin-RPE was diluted to a 1x concentration with Bio-Plex assay buffer and 50 µl were added to each well and incubated as before.
After washing to remove the unbound Streptavidin-RPE the beads (minimum 50 beads per cytokine) were analyzed using the Luminex 100 instrument. Luminex 100 monitored the spectral properties of the beads while simultaneously measuring the amount of fluorescence associated with R-phycoerythrin. Raw data (mean fluorescence intensity MFI) were analyzed by Bio-Plex Manager 4.1 software.

4.2.10 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR LIGAND ACTIVITY MEASUREMENT WITH TRANSFECTION

In transfection, foreign DNA is introduced into eukaryotic cells like HeLa, COS/7 and SZ95 cells. This is most often done as an instance of transient transfection, in which the transfected gene is expressed only transiently, that is in only the cells to which it was originally inserted and only for a short period of time. During cell mitosis the transient gene disappears.

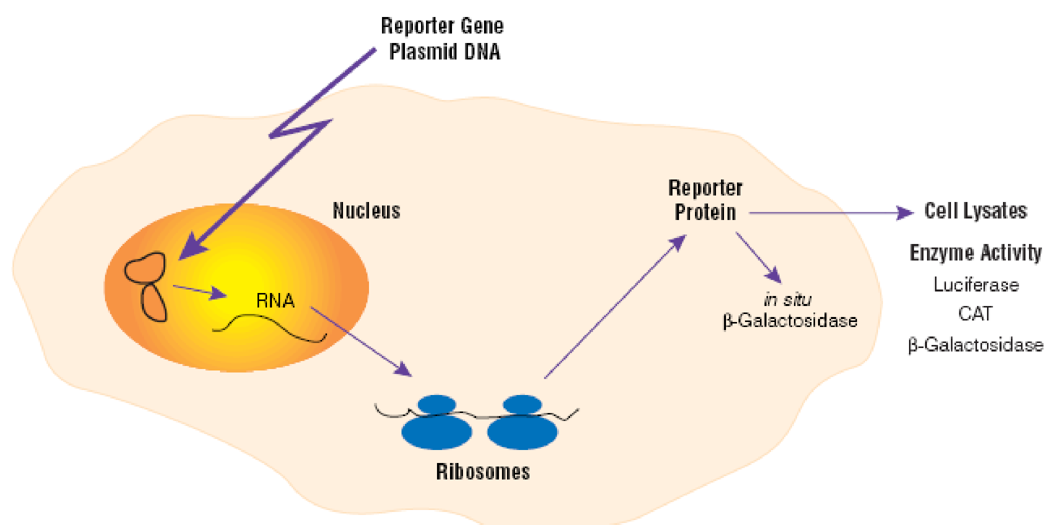


Figure 14 - Receptor gene system: PPARα, β/δ, γ_2 were transiently transfected in cells. Cells express more PPARα, β/δ, γ_2 which were activated by PPARα, β/δ, γ_2 ligands. The receptor activation was measured by luciferase enzyme activity.

4.2.10.1 TRANSIENT TRANSFECTION WITH LIPOFECTAMINE

The original method for cationic lipid-mediated DNA transfection was improved in 1993 by replacing the monocationic lipid reagent with lipofectamine, a polycationic reagent. The reaction was based on an ionic interaction of DNA and liposomes to form a complex. This complex can deliver functional DNA into cultured cells [40].

Transfection procedure:

With a coactivator dependent receptor ligand assay several natural compounds have been screened. Twelve-well culture plates were seeded with COS/7 or HeLa cells at a confluency of 50 - 60%. Incubation was overnight with DMEM medium and 10% FBS (fetal bovine serum) at 37°C, 5% CO_2. The following day, the medium was replaced and cells were transfected with empty expression vectors (pSG5) and vectors containing PPARα, β, γ_2.
The transfection preparation began with mixing and incubating lipofectamine (2 µl/well), Optimem (50 µl/well), renilla (5 ng/well) and PPRE (250 ng/well).
In another tube PPAR or pSG5 expression vector (400 ng/well) and Optimem (50 µl/well) were mixed.

655 ng DNA total/well = 250 ng PPRE.TK.Luc/well + 5 ng pTK-renilla/well + 400 ng PPAR or pSG5 empty vector/well

The transfection mix was pooled carefully with DNA mixture, then gently homogenized and incubated for 20 min at room temperature without shaking. Cells were washed with DMEM medium without serum/antibiotics before transfection. DMEM medium without serum/antibiotics (500 µl) was added to the transfection mixture (DNA/lipofectamine) and 600 µl/well were distributed. Cells were transfected

in triplicates. After 3 h incubation at 37°C, 5% CO_2 medium was replaced with DMEM with serum for 2 h.

In the meantime DMEM without serum and with ligands or compounds were prepared. COS/7 or HeLa cells were treated with 600 µl/well medium compositions. After 18 to 20 hours of culturing, cells were washed once with PBS. Lysis buffer (250 µl) was added and plates were agitated at 4°C during 15 min on a shaker. Then cells were scraped and collected. Cell lysate (10 µl) was transferred to 96-well opaque plates.
Luciferase and renilla activity were measured 24 h after transfection using the Dual-Luciferase Reporter Assay System, according to the manufacturer's instructions. Determination of normalized luciferase activity was plotted as fold activation relative to untreated cells. All experiments were performed in triplicates in at least three or more independent experiments.

4.2.10.2 TRANSIENT TRANSFECTION OF SZ95 SEBOCYTES

Transient transfection assays were performed using superfect transient reagent. This reagent assembles DNA into compact structures, to optimize the entry of DNA into SZ95 sebocytes. Once inside, superfect reagent buffers the lysosomes after fusion with the endosome, leading to pH inhibition of lysosomal nucleases and stability of superfect-DNA complexes.
Chen et al. observed that sebocytes cultured with serum free medium produce more lipids than those cultured with medium containing 10% fetal bovine serum. However, the exact actions of ligands on lipid metabolism are relatively complex and vary in different cellular systems [19].

<u>Preparation procedure:</u>

Sebocytes were trypsinized and transferred into 12-well culture plates, and incubated for 24 h to reach 40 - 50% (200,000 cells/well) confluency.

Total DNA (1.5 µg/well) (PPRE, pSG5 expression vectors and pSG5 expression vectors containing PPARα, δ, γ_2) was diluted in OptiMEM to a volume of 97 µl/well. Then 3 µl/well of superfect transfection reagent was added to the DNA solution. The samples were mixed and incubated for 5 - 10 min at room temperature to allow the complex formation.
While complex formation took place, growth medium was aspirated from the plates and cells were washed with 2 ml of PBS. Then 400 µl/well of Sebomed with 10% FCS without lipids was added to the reaction tube containing the transfection complexes. The transfection complex was mixed and immediately transferred to the SZ95 sebocytes for 2 h at 37°C, 5% CO_2. Then the medium was removed and cells were washed with 2 ml/well PBS. Fresh medium with compounds was added and sebocytes were treated and incubated for 24 h at 37°C, 5% CO_2.
At the indicated time, cells were rinsed with phosphate buffer and lysed in reporter lysis buffer. Then firefly and renilla activity were measured with the Promega dual reporter kit, according to the manufacturer's instructions. PPAR activation was absorbed by measurement of luciferase activity. All assays were performed in triplicates in 12-well plates, and the results were analyzed with a t-test.

4.2.10.3 BACTERIAL TRANSFORMATION

Bacterial transformation is a technique to introduce a foreign plasmid into bacteria. This method is based on the natural function of plasmid: to transfer genetic information vital to the survival of bacteria. A plasmid is a small circular piece of DNA that contains important genetic information for the growth of bacteria.

Preparation procedure:

Following the preparation procedure transformation of approximately 10^8 bacteria with 1 µg of pUC should give rise to 1-5 10^6 colonies.
Frozen competent *Escherichia coli (E. coli)* cells were thawed on ice, treated with beta-mercaptoethanol, mixed with a transforming plasmid, briefly heat shocked, and then transferred into 500 ml of LB medium with 50 µg/l ampillicin. The *E. coli* culture was grown overnight at 37°C with vigorous shaking (300 rpm).
The bacteria were then transferred into a sterile centrifuge bottle and spinned in a pre-cooled centrifuge (3000 rpm), for about 20 min, at 4°C; the supernatant was discarded.

For purification of plasmid DNA, Qiagen Mini- and Maxi-prep columns were used according to the manufacturer's instructions, as follows:
The plasmid purification was based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to an anion exchange resin under appropriate low salt and pH conditions. RNA, proteins and low molecular weight impurities were removed by medium salt wash. Plasmid DNA was eluted in a high salt buffer and then concentrated and desalted.
The bacterial pellet was resuspended in 10 ml of buffer P1. Immediately 10 ml of buffer P2 was added and mixed thoroughly but gently by inverting the tube several times, and incubated at room temperature for 5 min. Chilled buffer P3 (10 ml) was added and mixed as before. The sample was incubated on ice for 20 min and then centrifuged at 12000 rpm for 15 min; the supernatant was removed and stored on ice. A Qiagen-tip 500 was equilibrated with 10 ml of buffer QBT, and the supernatant was applied to the column and allowed to drain through under gravity. The column was washed twice with 30 ml of buffer QC. DNA was eluted with 15 ml of buffer QF, and precipitated by addition of 10.5 ml of isopropanol. The DNA pellet was washed with 5 ml ethanol of 70%. Finally the dried pellet, free of residual ethanol, was resuspended in 500 µl of deionized water. The concentration of DNA was determined by measuring the absorbance at 260 nm of a diluted sample. DNA samples were stored at -20°C.

4.2.11 STATISTICAL EVALUATION

Values represent the mean values ± standard deviation (SD) of at least three experiments. Statistical significance was calculated by the student's t-test. Mean differences were considered to be significant when ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *). Data and graphs were produced with MS Excel 2000.

5 RESULTS

5.1 SCREENING FOR ACTIVE COMPONENTS TO REGULATE LIPID SYNTHESIS

A new screening model using the human SZ95 sebaceous gland cell line was established in order to find new active compounds which affect the lipid synthesis in these cells. This model allowed measuring neutral lipids, polar lipids and cell vitality in one experiment for each well.

Table 14 - Active compounds from SZ95 sebocyte screening

Source	Name	Conc.	LA, AA pre-treatment				without pre-treatment			
			FDA	PL	NL in %	NL	FDA	PL	NL in %	NL
Microbial organism	Biolib P 75	1 µg/ml	-	-	88	↓*	↓*	↓*	77	↓**
Microbial organism	Biolib P 50	1 µg/ml	-	↓*	86	↓*	-	↓**	76	↓**
Microbial organism	Biolib P 90	1 µg/ml	-	↑*	127	↑*	↓*	↑**	108	-
Microbial organism	Biolib P 51	1 µg/ml	-	↓**	76	↓**	↓*	↓**	78	↓**
Bothrops Moojeni. Protein fractions	Botmo GF 1	1 µg/ml	-	-	117	↑*	-	-	114	↑*
Bothrops Moojeni. Protein fractions	Botmo GF 2	1 µg/ml	-	-	127	↑**	-	↑*	135	↑**
Bothrops Moojeni. Protein fractions	Botmo GF 3	1 µg/ml	-	-	119	↑*	-	-	111	-
Bothrops Moojeni. Protein fractions	Botmo GF 4	1 µg/ml	-	-	137	↑**	-	-	108	-
Bothrops Moojeni. Protein fractions	Botmo GF 5	1 µg/ml	-	-	152	↑**	-	-	131	↑***
Bothrops Moojeni. Protein fractions	Botmo GF 6	1 µg/ml	-	-	165	↑***	-	-	125	↑**
Bothrops Moojeni. Protein fractions	Botmo GF 7	1 µg/ml	-	↑*	215	↑***	-	↑*	154	↑**
Bothrops Moojeni. Protein fractions	Botmo GF 8	1 µg/ml	-	↑*	199	↑**	-	↑*	240	↑***
Bothrops Moojeni. Protein fractions	Botmo GF 9	1 µg/ml	-	↑*	240	↑***	-	↑*	250	↑***
Bothrops Moojeni. Protein fractions	Botmo GF 10	1 µg/ml	-	↑*	217	↑***	-	↑**	235	↑***
Bothrops Moojeni. Protein fractions	Botmo GF 11	1 µg/ml	-	↑*	202	↑***	-	↑*	173	↑***
Bothrops Moojeni. Protein fractions	Botmo GF 12	1 µg/ml	-	↑*	215	↑***	-	↑*	180	↑***
Bothrops Moojeni. Protein fractions	Botmo GF 13	1 µg/ml	-	↑*	168	↑***	-	↑*	194	↑***
Bothrops Moojeni. Protein fractions	Botmo GF 14	1 µg/ml	-	↑*	136	↑***	-	↑*	166	↑***
Bothrops Moojeni. Protein fractions	Botmo GF 15	1 µg/ml	-	↑*	205	↑***	-	-	161	↑***
Peptide	Tat-GLY-HIS-LYS-OH	0.1 µg/ml	-	↓*	84	↓**	-	-	83	↓**
Peptide	H-GLY-HIS-LYS-OH	0.1 µg/ml	-	-	83	↓**	-	-	102	-
Peptide	Cu GLY-HIS-LYS-OH	0.1 µg/ml	-	-	90	↓*	-	-	90	↓*
Plant extract	Prunus persica	1 µg/ml	-	-	111,5	↑**	-	-	114	↑*
Plant extract	Jatropha gossypifolia	1 µg/ml	-	-	83	↓**	-	-	76	↓***
Plant extract	Vitex agnus castus	0.1 µg/ml	-	-	82	↓**	-	-	108	-
Plant extract	Dioscorea villosa	0.1 µg/ml	-	-	119	↑**	-	-	122	↑**

Legend: NL neutral lipids, PL polar lipids, AA arachidonic acid, LA linoleic acid, glycyl-histidyl-lysine (Gly-His-Lys), FDA fluoresceine diacetate, (-) no significant effect. Yellow labeled compounds are considered for further studies. Data are shown as mean ± standard deviation of triplicates (n = 9). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *) was considered statistically significant. Lipid stimulation was marked with ↑ and lipid inhibition with ↓. Untreated control cells correspond to 100% neutral lipids.

Table 14 displays 26 active substances of plant extracts, snake venom gel filtration fractions, bacteria extracts and synthetic peptides, which lead to lipid stimulation or reduction in sebocytes. The SZ95 sebocyte screening model has been carried out with two different methods.

One method was sebocyte pre-treatment with arachidonic acid and linoleic acid which increases SZ95 sebocyte neutral lipid production and was used to find compounds which inhibit lipid production. After pre-lipid stimulation some

compounds, such as *Bothrops moojeni* venom gel filtration fractions, additionally stimulated the increased production of neutral and polar lipids. Compounds like glycyl-histidyl-lysine (GHK) and their derivatives, *Jatropha gossypifolia*, *Vitex agnus castus* reduced lipid production in SZ95 sebocytes.
The other method tested the same compounds without arachidonic or linoleic acid pre-treatment. This method showed similar results compared to the experiments with arachidonic acid or linoleic acid pre-treatment.
The remaining 175 compounds, which are not listed in Table 14 lead to no significant effects on neutral lipid regulation or were cytotoxic (cf. chapter 4.1.2).

The plant extract *Jatropha gossypifolia* inhibited neutral lipid synthesis by about 17 - 24% ($p < 0.001$ - $p < 0.01$). *Vitex agnus castus* blocked lipid expression by 18% ($p < 0.01$); however this effect was only observable with a fresh extract. *Prunus persica* and *Dioscorea villosa* boosted neutral lipid expression by 17 - 19% ($p < 0.01$) in cells.
Bacteria extracts stimulated production of neutral lipids up to 10 - 27% in sebocytes, but a cell vitality reduction ($p < 0.05$) was also measurable, which is an indicator for cytotoxicity. In arachidonic and linoleic acid pre-stimulated SZ95 sebocyte cell vitality loss was not detectable.
The three derivatives of GHK peptides reduced lipids in arachidonic or linoleic acid pre-treated SZ95 sebocytes. GHK and GHK derivatives inhibited pre-stimulated neutral lipids about 10 - 17%; $p < 0.01$ - $p < 0.05$. In non-stimulated cells GHK treatment had no measurable effect on SZ95 sebocyte lipids.
Especially noticeable were the snake venom gel filtration fractions from *Bothrops moojeni*. All gel filtration fractions stimulated neutral lipid production in SZ95 sebocytes significantly up to 140%; $p < 0.001$ without toxic effects. Even when sebocytes were pre-treated with arachidonic acid or linoleic acid, a lipid increase was observed.

The objective of this screening was to identify active compounds influencing lipid metabolism in SZ95 sebocytes. Because *Bothrops moojeni* venom gel filtration fractions lead to the most promising results, the lipogenic influence of *Bothrops moojeni* venom gel filtration fractions were the main focus of this work.
Three Botmo gel filtration fractions 11, 12 and 15, from the low molecular weight range were selected as the most interesting ones, showing best results in lipid stimulation. Botmo GF 11, 12 and 15 elute lately from the gel filtration column, having a lower molecular weight than earlier eluted fractions. Low molecular weight Botmo GF fractions are better suited for a complete identification of the fraction and for new possible topically used ingredients.
Additionally, the inhibition of lipid production was investigated using GHK and *Vitex agnus castus* extract.

5.1.1 LIPID REGULATION BY *BOTHROPS MOOJENI* VENOM

The previous screening results showed for the first time ever that *Bothrops moojeni* venom fractions activated lipid synthesis in SZ95 sebocytes. In order to identify the right dose-response relationship of Botmo gel filtration fractions (Botmo GF) eight concentrations were tested. The three diagrams below show lipid stimulation in Botmo GF 11, Botmo GF 12 and Botmo GF 15 treated sebocytes (Figure 15).

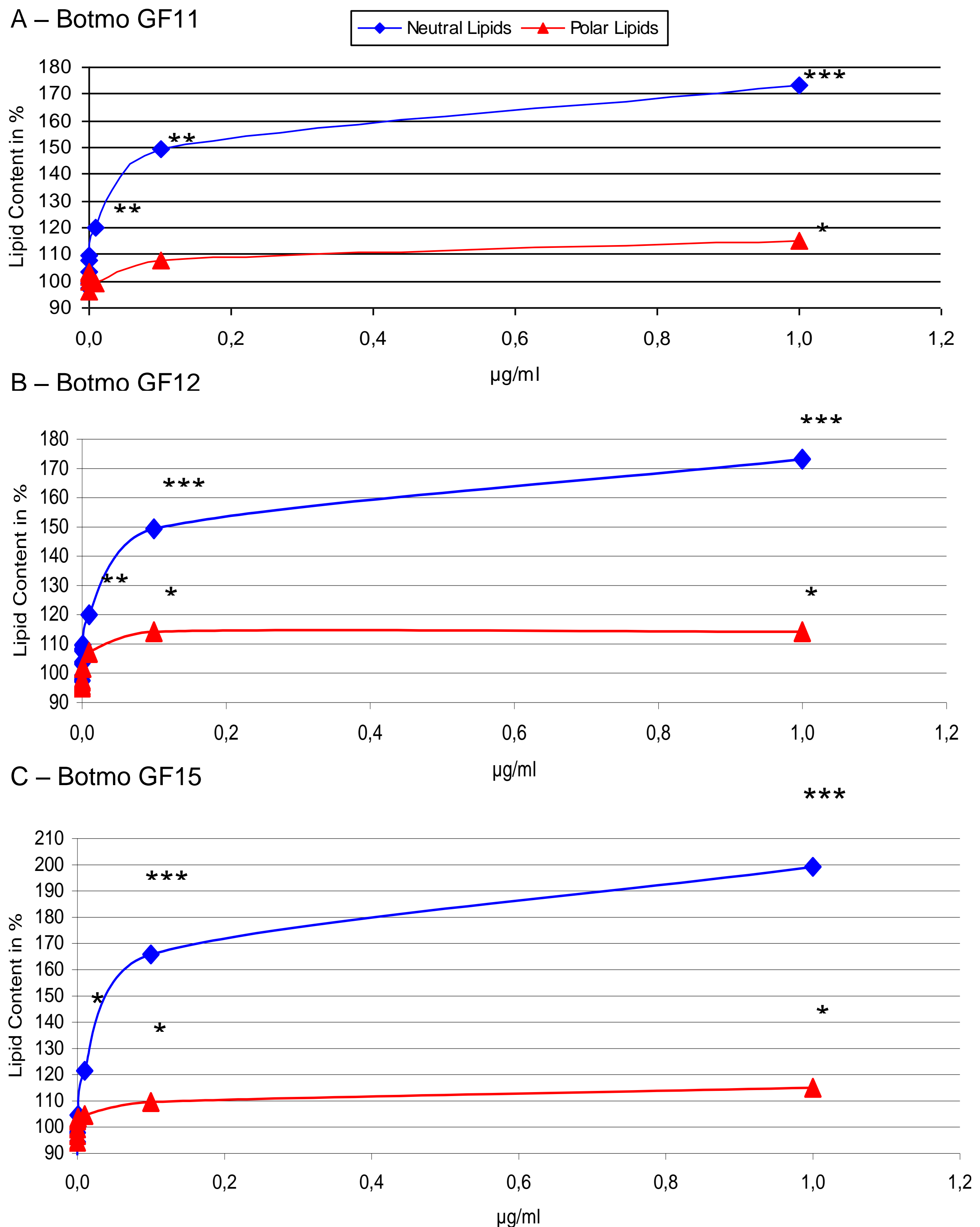


Figure 15 - Lipids in SZ95 sebocytes incubated with Botmo gel filtration fractions 11, 12 and 15. Sebocytes were seeded at a density of 40,000 cells/well in a 96-well plate and incubated for 24 h. On the next day SZ95 sebocytes without pre-treatment were treated with Botmo GF 11, Botmo GF 12 or Botmo GF 15 for 24 h in different concentrations. Neutral lipids (blue line ◆) and polar lipids (red line ▲) were measured with Nile Red dye. The fluorescence intensity of polar lipids and neutral lipids was shown in %. The fluorescence intensity of polar lipids is about 30,000 auto fluorescence units (AFU), neutral lipids about 100 AFU. Lipid production of control cells with no treatment was set to 100%. These data are significant compared to control cells. Data are shown as mean ± standard deviation of triplicates (n = 6). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

Graph A shows the measured lipid content of Botmo GF 11 treated SZ95 sebocytes. The concentration of Botmo GF 11 ranged from 0.0001 ng/ml to 1 µg/ml. At a concentration of 1 µg/ml neutral lipids were significantly increased to 173% ($p < 0.001$), at 0.1 µg/ml to 150% ($p < 0.001$) neutral lipids and at 0.01 µg/ml to 120% ($p < 0.01$) neutral lipids. Lower concentrations showed no significant effects. Only at 1 µg/ml polar lipid increase in SZ95 sebocytes demonstrated a low significance ($p < 0.05$).
The measured lipid content of Botmo GF 12 treated SZ95 sebocytes was shown in graph B. A concentration of 1 µg/ml resulted in 173% neutral lipid stimulation ($p < 0.001$), 0.1 µg/ml 150% neutral lipid stimulation ($p < 0.001$) and 0.01 µg/ml 120% neutral lipid stimulation ($p < 0.05$). Lower concentrations showed no significant effects. From 1 µg/ml to 0.1 µg/ml polar lipid synthesis was increased in SZ95 sebocytes with a low significance ($p < 0.05$).

Graph C displays Botmo GF 15 treated SZ95 sebocyte results. The neutral lipid production in SZ95 sebocytes was increased by 200% ($p < 0.001$) after stimulation with 1 µg/ml Botmo GF 15. A concentration of 0.1 µg/ml Botmo GF 15 stimulated 166% ($p < 0.001$) neutral lipids and 0.01 µg/ml 122% ($p < 0.05$) neutral lipids. Lower concentrations showed no significant effects. At a concentration of 0.1 µg/ml polar lipid increase in SZ95 sebocytes was significant ($p < 0.05$). For all these three figures the production of neutral lipids was more strongly stimulated than that of polar lipids.

When neutral lipid production increased to significantly high levels, polar lipids were sometimes rising as well and reached a maximum of 118%; $p < 0.05$. Neutral lipid amounts were more significantly increased than polar lipids. Additionally, no toxic effects could be observed with LDH cytotoxicity assays (data not shown).
Botmo GF 11, 12 and 15 showed comparable results on lipid synthesis and vitality in SZ95 sebocytes.

5.1.2 APOPTOSIS – MEASUREMENT OF BOTMO GF11, 12 AND 15 TREATED SZ95 SEBOCYTES

Increased cell volume, accumulation of lipid droplets in the cytoplasm and nuclear degeneration are phenomena indicating terminal differentiation. Lipid stimulation in this range can be related to a late differentiation phase of SZ95 sebocytes. This differentiation state can lead to holocrine secretion and cell death [173]. SZ95 sebocytes treated with *Bothrops moojeni* venom gel filtration fractions demonstrated increased neutral lipid accumulation, good cell vitality and no cytotoxic effects in all used concentration ranges.
To verify these results and to rule out morphological processes of controlled cellular self-destruction (apoptosis), caspases 3/7 levels were measured (cf. chapter 4.2.8).

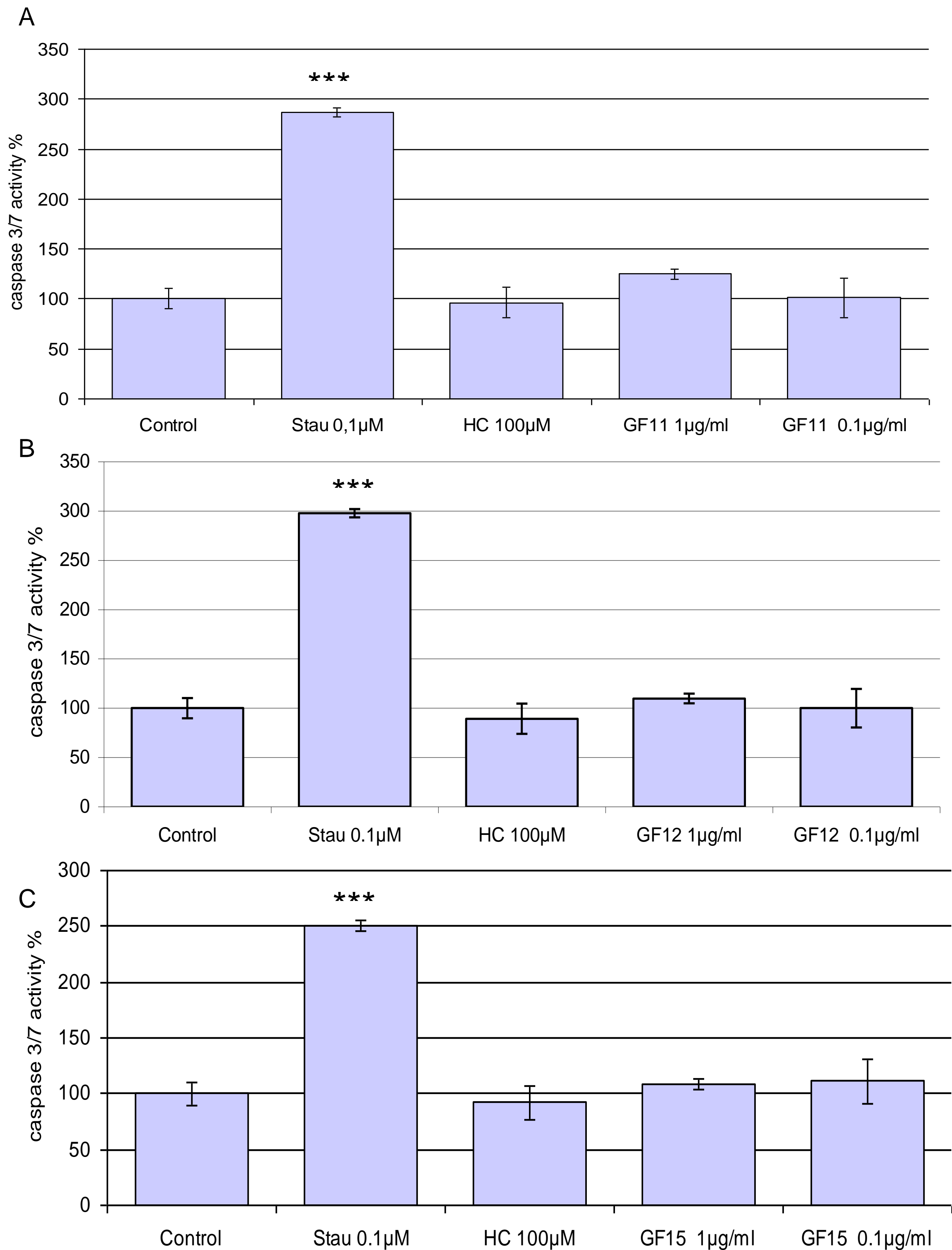


Figure 16 - Caspase-3/7 activity measurement of SZ95 sebocytes treated with Botmo gel filtration fractions 11, 12 and 15. SZ95 Sebocytes were seeded at a density of 40,000 cells/well in a 96-well plate and incubated for 24 h. On the next day cells were treated with Botmo GF 15 in two concentrations, hydrocortisone (HC) and a positive control staurosporin 10^{-7} M (Stau). Caspase-3/7 measurement was carried out all 30 minutes for 18 h. Graph A shows Botmo GF 11, Graph B illustrates Botmo GF 12 and Graph C displays Botmo GF 15 treated SZ95 sebocyte results. All measurements show good consensus with no important changes. The diagram shows the final results. Data are shown as mean ± standard deviation of triplicates (n = 3). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

Figure 16 shows results of SZ95 sebocytes which were treated with Botmo GF 11, 12, 15 and staurosporin, Staurosporin induced apoptosis significantly ($p < 0.001$) and served as a control. No caspase-3/7 releases were measurable in all SZ95 sebocytes treated with the three Botmo GF fractions in used concentration ranges.

Parallel to the caspase-3/7 experiment, measurements of neutral, polar lipids and cell vitality were performed. Botmo GF 15 at 1 μg/ml and 0.1 μg/ml produced significant neutral lipids up to approximately 200% ($p < 0.001$) or 166% ($p < 0.001$), respectively as shown in Figure 15.
To confirm the assumption that only *Bothrops moojeni* venom gel filtration fractions 11, 12 and 15 are able to significantly stimulate neutral lipids, all buffers were included in this test. However, this test demonstrated no caspase-3/7 activity for Botmo GF 11, 12, 15, buffers and hydrocortisone.

5.1.3 ACTION OF BOTMO GF 15 ON LIPID SYNTHESIS IN SZ95 SEBOCYTES

All Botmo GF 5 to 15 demonstrated highly significant neutral lipid accumulation in SZ95 sebocytes, but no toxic effects. Based on these results more investigations on *Bothrops moojeni* venom fractions followed. Botmo GF 15 was used for further separation steps and various measurements.

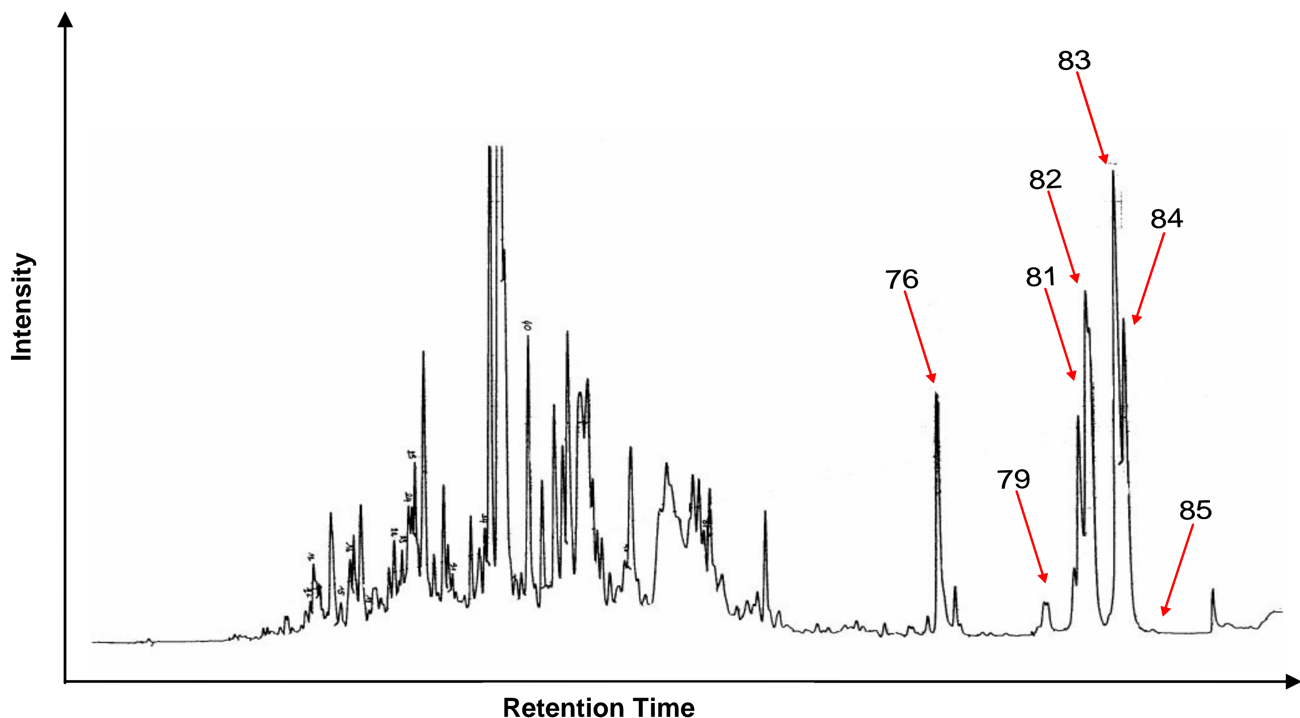
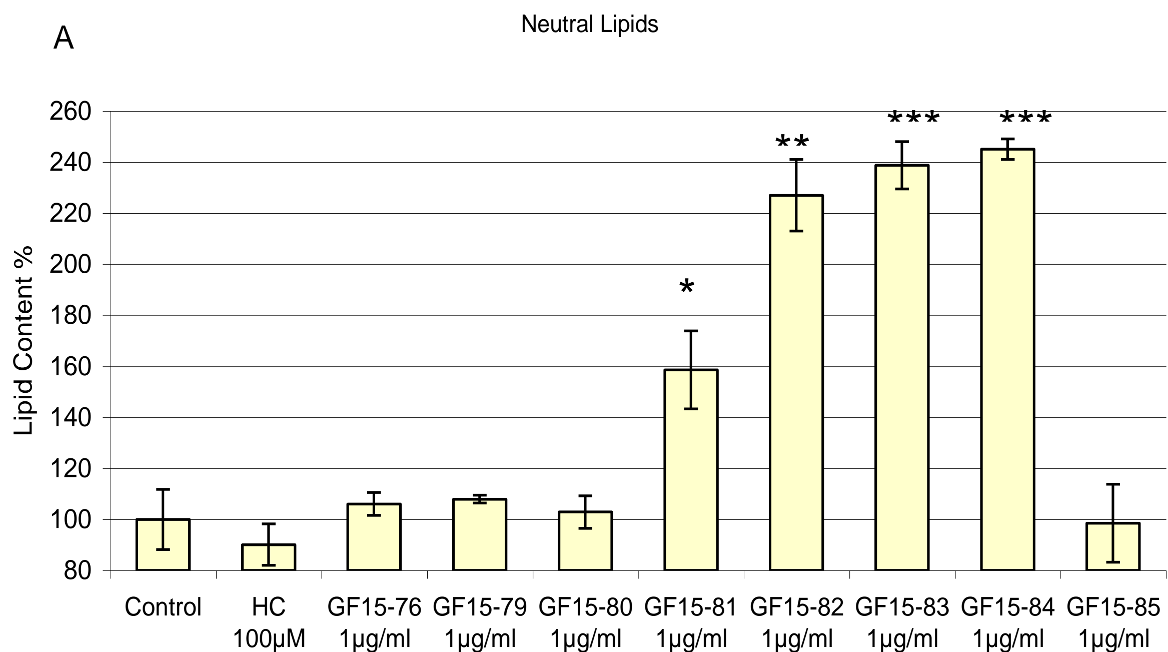


Figure 17 - Semi preparative HPLC profile of Botmo GF 15. The desalted Botmo GF fraction 15 was processed by RP-HPLC on an Waters Alliance 2690 System using a semi preparative HPLC column (Vydac #218TP510 protein & peptide C_{18}, 10 mm x 250 mm) (cf. chapter 4.2.1.4) [115].

The semi preparative HPLC showed that Botmo GF 15 fraction still contained a lot of compounds which await their characterization (Figure 17). Since the HPLC

chromatograms showed relatively well separated fractions, they have been chosen for further experiments. HPLC fractions were collected and lyophilized (c.f. 4.1.2; page 27). The collected fractions were tested in 1 µg/ml concentrations using the *in vitro* SZ95 sebocyte model.

Botmo GF fraction 15 subfractions 1 to 80 were tested on SZ95 sebocytes but did not lead to significant increase of lipid production. As seen in Figure 18, Botmo GF 15 subfractions 76 to 80 showed no neutral and polar lipid stimulation in SZ95 sebocytes. However Botmo GF 15-81 led to a lipid increase of 160% ($p < 0.05$). Botmo GF 15 subfraction 82 stimulated neutral lipids in sebocytes to 223% ($p < 0.01$), Botmo GF 15-83 and Botmo GF 15-84 about 240% - 245% ($p < 0.001$). In these tests polar lipids were increased up to 140% ($p < 0.05$), but neutral lipids were again more strongly stimulated.



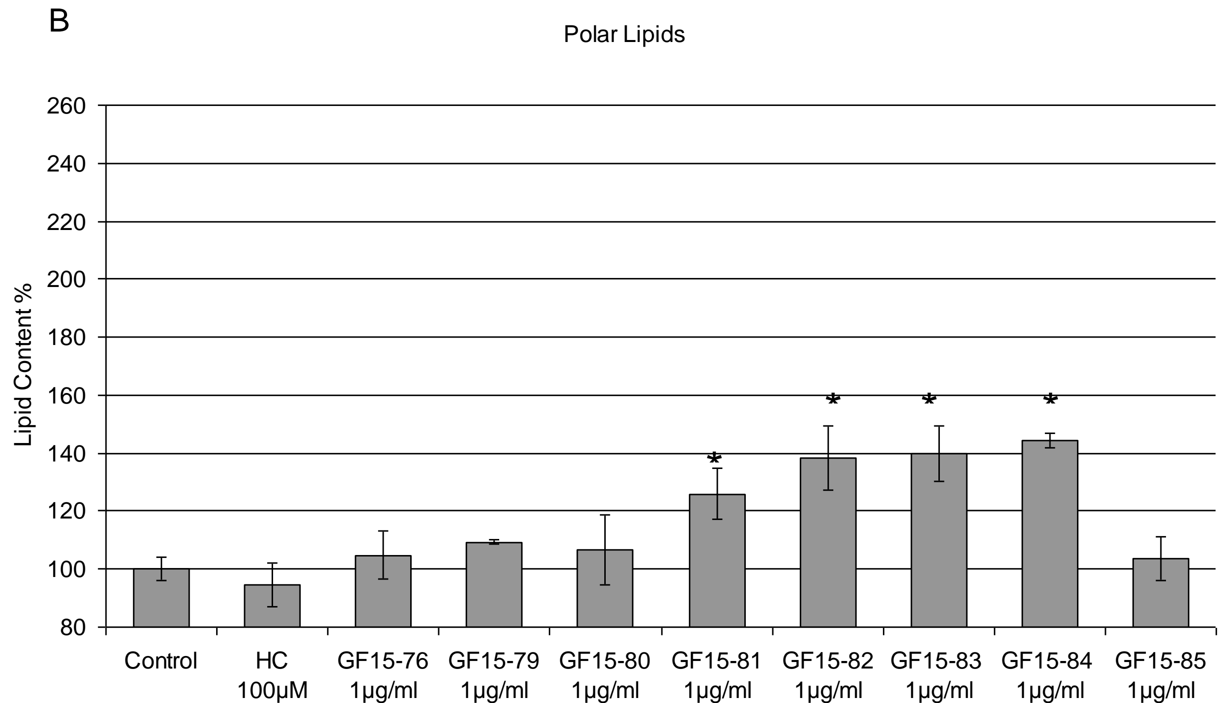


Figure 18 - Lipids in SZ95 sebocytes incubated with Botmo gel filtration fraction 15. SZ95 sebocytes were seeded at a density of 40,000 cells/well in a 96-well plate and incubated for 24 h. On the next day cells were treated with hydrocortisone (HC) and Botmo GF 15 HPLC fractions (see Semi preparative HPLC profile). Neutral lipids (yellow bars) and polar lipids (grey bars) were measured with Nile Red dye. Lipid production of pos. control cells with no treatment was set to 100%. Data are shown as mean ± standard deviation of triplicates (n = 3). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

Semi preparative RP-HPLC separation of Botmo GF 11, 12 and 15 resulted in many different compounds (Figure 17). The compounds, which stimulated lipogenesis in SZ95 sebocytes, eluted late from HPLC column (Botmo GF 15 subfractions 82 - 84). Two selected fractions of Botmo GF 11-101, Botmo GF 11-117 and all active GF 15 (GF 15-82 to GF 15-84) fractions were used for further investigations. There was not enough time to test Botmo GF 12.

5.2 GEL FILTRATION FRACTION OF *BOTHROPS MOOJENI* VENOM CONTAINING PHOSPHOLIPASE A_2

According to gel filtration measurements, Botmo GF 1 - 18 were divided into high (Botmo GF 1 - 8) and low (Botmo GF 9 - 18) molecular weight components. Compounds in the molecular mass range of $sPLA_2$ (13 - 15 kDa) could be identified in the low molecular weight Botmo GF 9 - 15 [115].

Two tests were performed, one to identify the $sPLA_2$ IIA and the other one to measure the PLA_2 enzymatic activity.
All fractions Botmo GF 15-76 to 85 were found to be $sPLA_2$ IIA as tested by an immunometric assay. Botmo GF 15-81 to 84 (1 μg/ml) which were able to increase ($p < 0.001$ - $p < 0.05$) neutral lipids significantly, showed $sPLA_2$ enzymatic activity (PLA_2 activity assay). Furthermore, fractions Botmo GF 15-76, Botmo GF 15-79, Botmo GF 15-80 and Botmo GF 15-85 also comprise $sPLA_2$ structure, showing however no detectable PLA_2 enzymatic activity.

Additionally, two fractions of Botmo GF 11 were tested. As expected both fractions Botmo GF 11-101 and GF 11-117 were compounds of the PLA_2 family, but only Botmo GF 11-117 was enzymatically active ($p < 0.01$). Snake venom fraction Botmo GF 11-101 with no detectable enzymatic activity significantly stimulated neutral lipid synthesis in SZ95 sebocytes (Table 15).

Table 15 - HPLC fractions and their phospholipase A_2 activity

HPLC Fraction	NL in %	Significance	$sPLA_2$ IIA	PLA_2 activity	Significance
GF15-76	115	-	Yes	0,09	-
GF15-79	111	-	Yes	0,007	-
GF15-80	111	-	Yes	0,016	-
GF15-81	160	*	Yes	0,235	*
GF15-82	223	**	Yes	1,894	***
GF15-83	240	***	Yes	1,980	***
GF15-84	245	***	Yes	1,865	***
GF15-85	100	-	Yes	.0,012	-
GF11-101	**150**	***	Yes	**0,034**	**-**
GF11-117	**310**	***	Yes	**2,619**	***

Legend: NL neutral lipids, sPLA2 IIA phospholipase type IIA, (-) no significant effect. Data are shown as mean ± standard deviation of triplicates (n = 3). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *)

The following HPLC chromatograms A and B show that enzymatically inactive PLA_2 eluted earlier from HPLC column. Fractions later in the elution showed enzymatic activity.

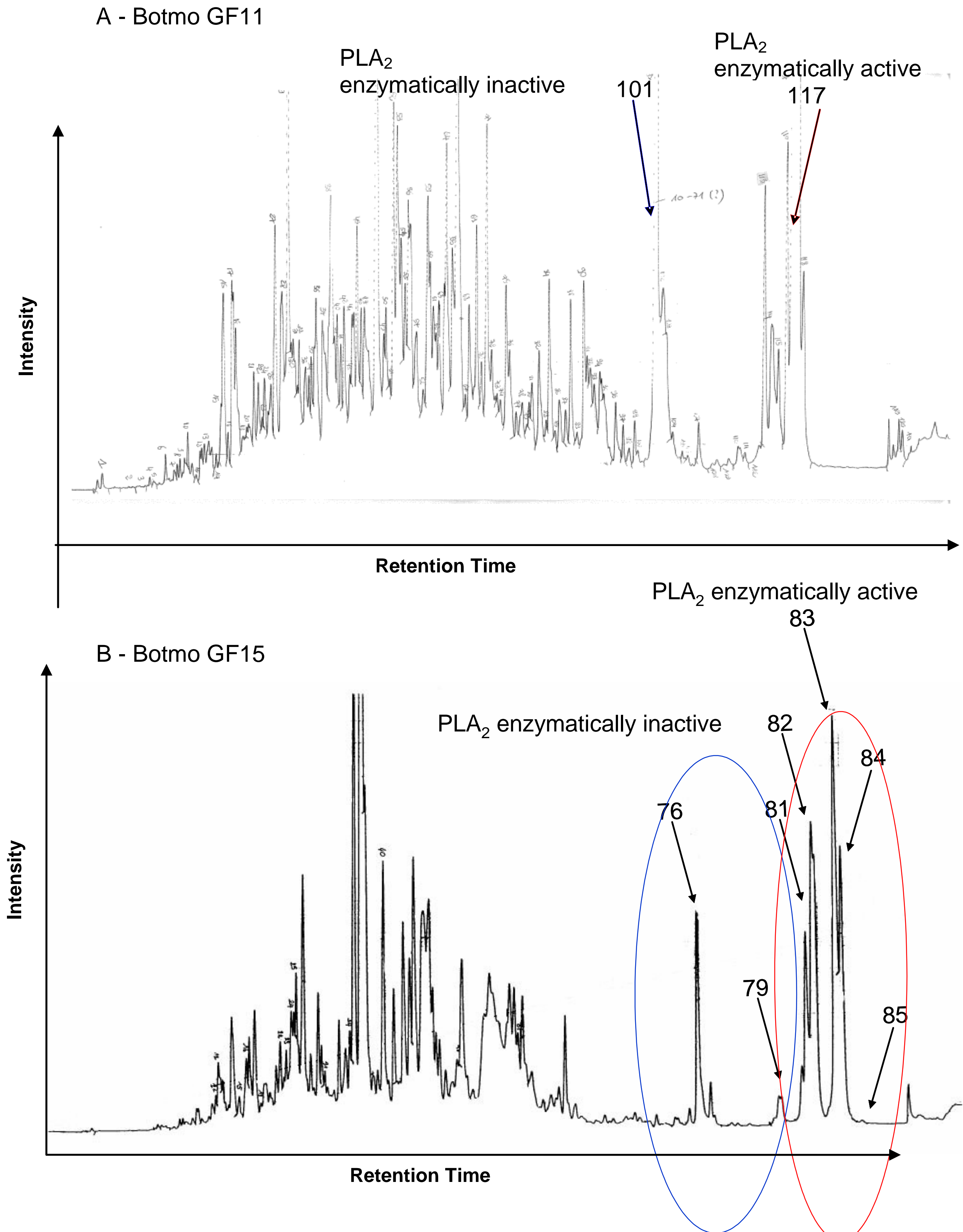


Figure 19 - HPLC chromatograms of Botmo GF 11 (A) and 15 (B) with PLA_2 active fractions. Blue area or arrow contains PLA_2-molecules which are enzymatically inactive. Red area or arrow shows PLA_2 enzymatically active molecules. These chromatograms are kindly provided by Anna Maria Perchuc.

After HPLC separation more material was available from Botmo GF 11-101 and GF 11-117 compared to Botmo GF 15-76 and Botmo GF 15-83 or Botmo GF 15-84 fractions. Because of their higher significant neutral lipid activity and more available material Botmo GF 11-101 ($p < 0.01$) and Botmo GF 11-117 ($p < 0.001$) were used for further investigation.

5.3 REGULATION OF LIPIDS IN SZ95 SEBOCYTES

5.3.1 LIPID STIMULATION IN BOTMO GF 11-101 AND BOTMO GF 11-117 TREATED SZ95 SEBOCYTES

Previous experiments in this work showed that Botmo GF 11-101 and Botmo GF 11-117 contain molecules which belong to the $sPLA_2$ family, but only Botmo GF 11-117 was identified to be PLA_2 enzymatically active. $sPLA_2$ of *Naja mossambica mossambica* snake venom were tested on SZ95 sebocytes to compare these two Botmo GF 11-101 and GF 11-117 fractions with a defined and identified snake $sPLA_2$.

$sPLA_2$ of *Naja mossambica mossambica* venom was used as positive control to compare with other $sPLA_2$ from snake venoms. After 24 h treatment of SZ95 sebocytes with Botmo GF 11-101, Botmo GF 11-117 and $sPLA_2$ of *Naja mossambica mossambica* venom (positive control) a significant increase of neutral and polar lipids was observed. Botmo GF 11-101 up regulates neutral lipid synthesis by 150% ($p < 0.01$). Botmo GF 11-117 leads to significantly higher expression of neutral lipids: 310% ($p < 0.001$). $sPLA_2$ from *Naja mossambica mossambica* snake venom affects neutral lipid expression by 290% ($p < 0.001$).
Botmo GF 11-117 and $sPLA_2$ from *Naja mossambica mossambica* snake venom significantly increased polar lipids ($p < 0.05$).

These secreted PLA_2 were compared with *honey bee* $sPLA_2$. *Honey bee* PLA_2 slightly stimulated neutral lipid production in SZ95 sebocytes, but was in all other experiments rather inactive compared to Botmo GF 11-101, Botmo GF 11-117 and $sPLA_2$ from *Naja mossambica mossambica* (data not shown).

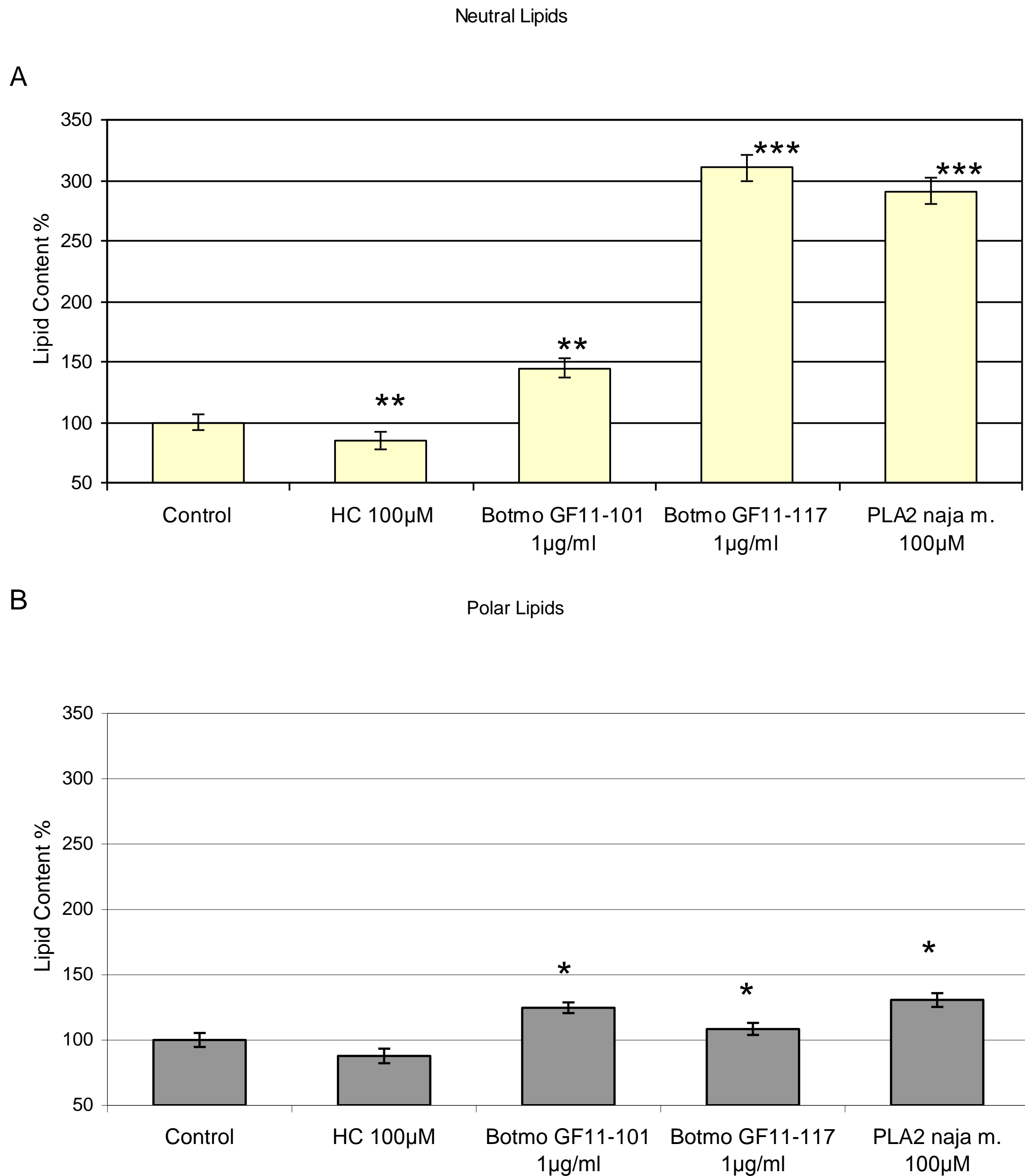


Figure 20 - Lipid content in Botmo GF 11-101, Botmo GF 11-117 and PLA_2 of *Naja mossambica mossambica* treated SZ95 sebocytes. SZ95 sebocytes were seeded at a density of 40,000 cells/well in a 96-well plate, incubated for 24 h. On the next day cells were treated with hydrocortisone (HC), Botmo GF 11-101, Botmo GF 11-117 and PLA_2 from *Naja mossambica mossambica* (PLA_2 naja m.). Data are shown as mean ± standard deviation of triplicates (n = 6). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

5.3.2 CYCLOOXYGENASE-2 INHIBITOR AND LIPOXYGENASE INHIBITOR EFFECT ON LIPID SYNTHESIS

The results so far suggested that Botmo GF11-101, Botmo GF11-117 and PLA_2 stimulated lipid synthesis by activating the arachidonic acid metabolism in SZ95 sebocytes. To inhibit the lipid synthesis by arachidonic acid, general LOX inhibitor (NDGA) and COX-2 inhibitors (NS398) were tested for their ability to suppress lipid production in SZ95 sebocytes.

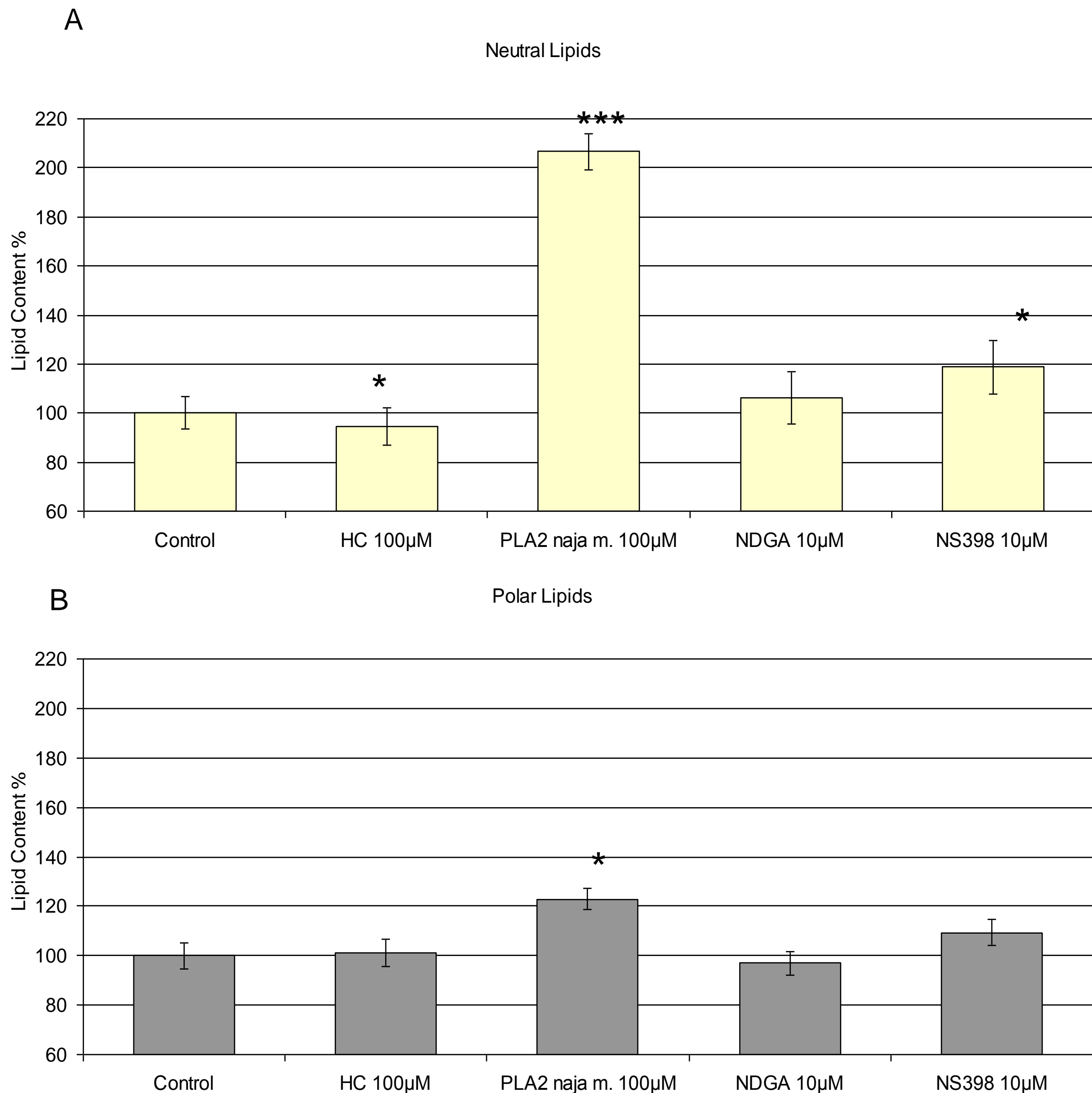
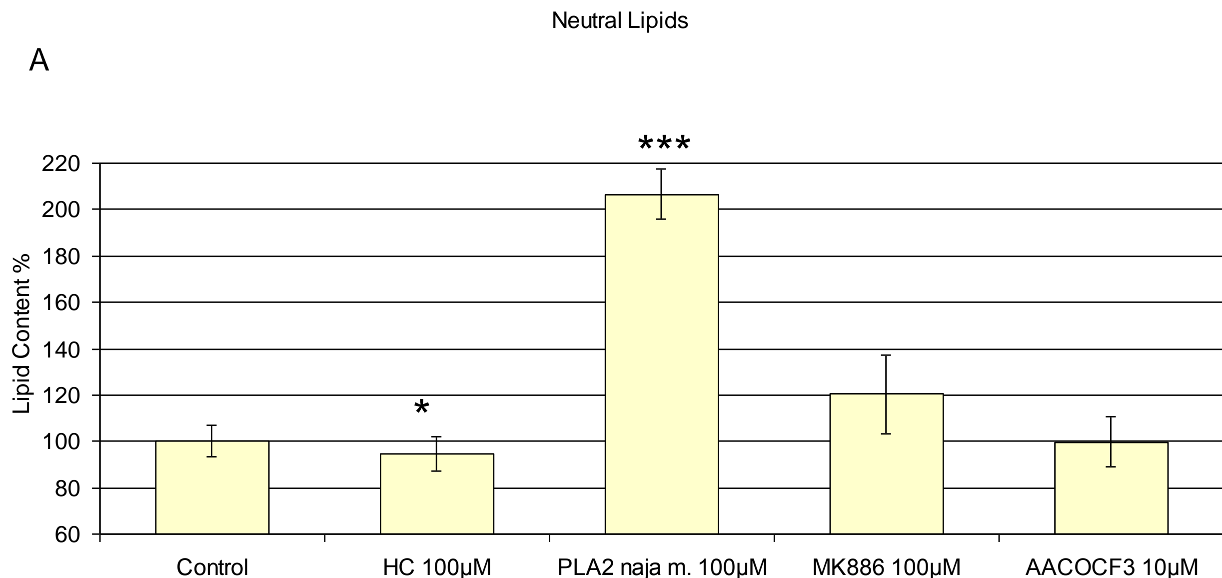


Figure 21 - Lipid content in general LOX inhibitor and COX-2 inhibitor treated SZ95 sebocytes. SZ95 sebocytes were seeded at a density of 40,000 cells/well in a 96-well plate and incubated for 24 h. On the next day cells were pre-stimulated with arachidonic acid and treated with hydrocortisone (HC), *Naja mossambica mossambica* $sPLA_2$ (PLA_2 naja m.), general LOX inhibitor (NDGA) and COX-2 inhibitor (NS398). Data are shown as mean ± standard deviation of triplicates (n = 3). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

After treatment of SZ95 sebocytes with COX-2 inhibitor significant stimulation of neutral lipids 120% ($p < 0.05$) was observed. The general LOX inhibitor did not stimulated neutral lipids significantly. None of these inhibitors blocked or reduced neutral or polar lipid synthesis in SZ95 sebocytes when compared to stimulated or inhibited control cells (Figure 21).

5.3.3 SZ95 SEBOCYTES TREATMENT WITH 5-LIPOXYGENASE INHIBITOR AND PHOSPHOLIPASE INHIBITOR

The 5-LOX inhibitor (MK886) and PLA_2 inhibitor ($AACOCF_3$) were tested for their influence on the lipid content in SZ95 sebocytes; however there was no significant change in neutral or polar lipid production in SZ95 sebocytes. In detail, MK886 demonstrated a low neutral lipid stimulation which was not significant and $AACOCF_3$ showed no effect compared to control cells.



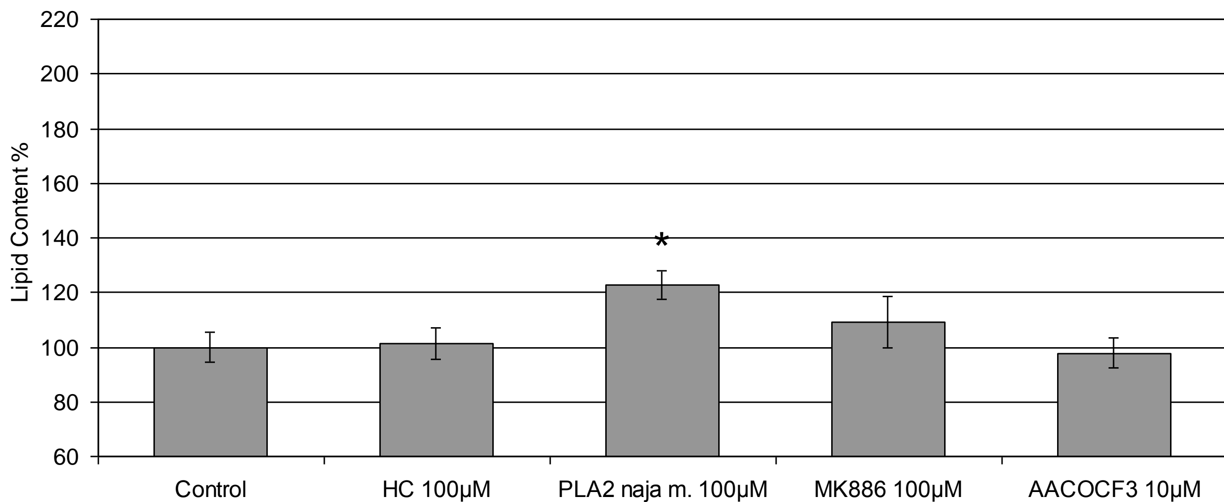


Figure 22 - Lipid content in 5-LOX and PLA_2 inhibitors treated SZ95 sebocytes. SZ95 sebocytes were seeded at a density of 40,000 cells/well in a 96-well plate and incubated for 24 h. On the next day cells were pre-stimulated with arachidonic acid and treated with hydrocortisone (HC), *Naja mossambica mossambica* $sPLA_2$ (PLA_2 naja m.), 5-LOX inhibitor (MK886) and PLA_2 inhibitor ($AACOCF_3$). Data are shown as mean ± standard deviation of triplicates (n = 3). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

5.3.4 PHOSPHOLIPASE PRE-STIMULATED SZ95 SEBOCYTES TREATED WITH CYCLOOXYGENASE-2 INHIBITOR, 5-LIPOXYGENASE INHIBITOR AND PHOSPHOLIPASE INHIBITOR

To get more knowledge about the increased neutral lipid synthesis in SZ95 sebocytes after PLA_2 treatment, sebocytes were pre-stimulated with *Naja mossambica mossambica* PLA_2 and 1 h later treated with Botmo GF 11-117, general LOX inhibitor (NDGA) and COX-2 inhibitor (NS398), 5-LOX inhibitor (MK886) and PLA_2 inhibitor ($AACOCF_3$). Additional lipid synthesis stimulation after Botmo GF 11-117, general LOX inhibitor (NDGA), COX-2 inhibitor (NS398) and 5-LOX inhibitor (MK886) was observed (Figure 23). However, under these circumstances Botmo GF 11-117 was not able to stimulate neutral lipids synthesis in SZ95 sebocytes significantly.

Treatment with PLA_2 inhibitor ($AACOCF_3$) showed not significant lipid suppression (5%) in SZ95 sebocytes.

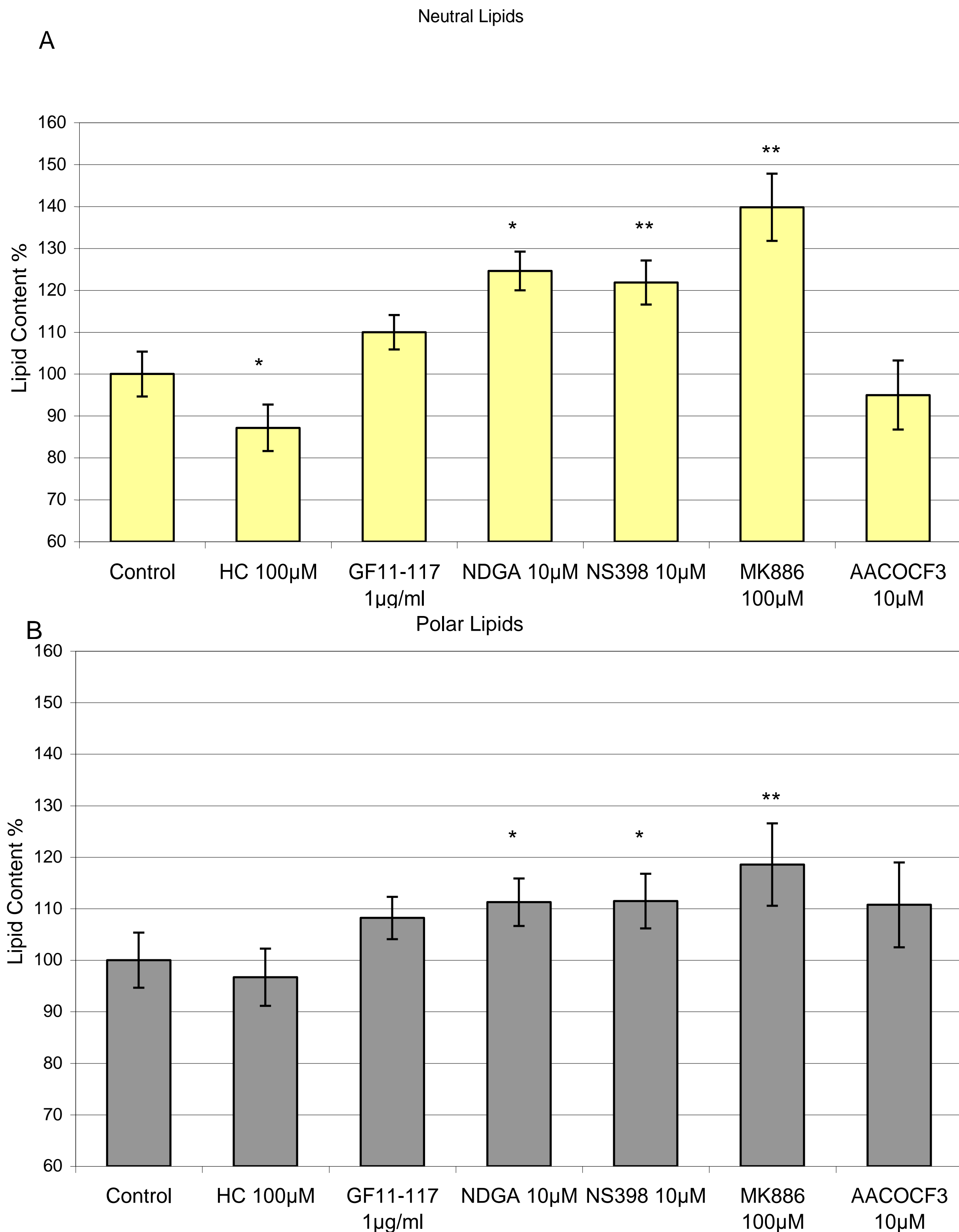


Figure 23 - Lipids in SZ95 sebocytes pre-incubated with Naja mossambica mossambica phospholipase. SZ95 sebocytes were seeded at a density of 40,000 cells/well in a 96-well plate and incubated for 24 h. On the next day cells were pre-treated with Naja mossambica mossambica $sPLA_2$ (PLA_2 naja m.), for 1 h, and then treated with hydrocortisone (HC), Botmo GF 11-117, general LOX inhibitor (NDGA) and COX-2 inhibitor (NS398), 5-LOX inhibitor (MK886) and PLA_2 inhibitor (AACOCF3). Neutral lipids (yellow bars) and polar lipids (grey bars) were measured with Nile Red staining. Lipid production of control cells with no treatment was set to 100%. Data are shown as mean ± standard deviation of triplicates (n = 3). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

5.3.5 LIPID REGULATION IN SZ95 SEBOCYTES BY GLYCYL-HISTIDYL-LYSINE

Results from the SZ95 sebocyte screening demonstrated that glycyl-histidyl-lysine (GHK) and its derivatives reduced neutral lipid synthesis significantly by 15% ($p < 0.01$ - $p < 0.05$). A measurable lipid decrease in SZ95 sebocytes of more than 10%, which is statistically significant, is interesting for further experiments.
Cu GHK, GHK and Tat-GHK reduced neutral lipid content in arachidonic or linoleic acid pre-stimulated SZ95 sebocytes ($p < 0.01$ - $p < 0.05$). Without pre-stimulation Cu GHK and Tat-GHK inhibited neutral lipid production by 10 - 13%. GHK had no effect on the lipid content in unstimulated SZ95 sebocytes (see Table 14). This result was comparable to earlier observations with hydrocortisone treatment [145].
Part of the study was to investigate if GHK is able to influence the production of neutral lipids and the expression of cytokines in SZ95 sebocytes, in a similar way as hydrocortisone.
In Figure 24, the effects of GHK on SZ95 sebocytes *in vitro* are shown. For this study SZ95 sebocyte lipid production was stimulated either with 0.1 mM linoleic acid or 0.01 mM arachidonic acid. Both fatty acids led to similar results. After 1 h pre-incubation with linoleic acid or arachidonic acid, cells were treated with hydrocortisone or GHK.
Treatment with GHK (1 mM) significantly inhibited pre-stimulated neutral lipid synthesis by 15% ($p < 0.01$). A GHK concentration of 0.1 mM showed no effects on neutral and polar lipids. No lipid inhibition or lipolytic effect on SZ95 sebocytes was measured without pre-stimulating neutral lipids. Cell viability and proliferation remained unchanged. After 24 h treatment no cytotoxicity was detected with LDH (data not shown).

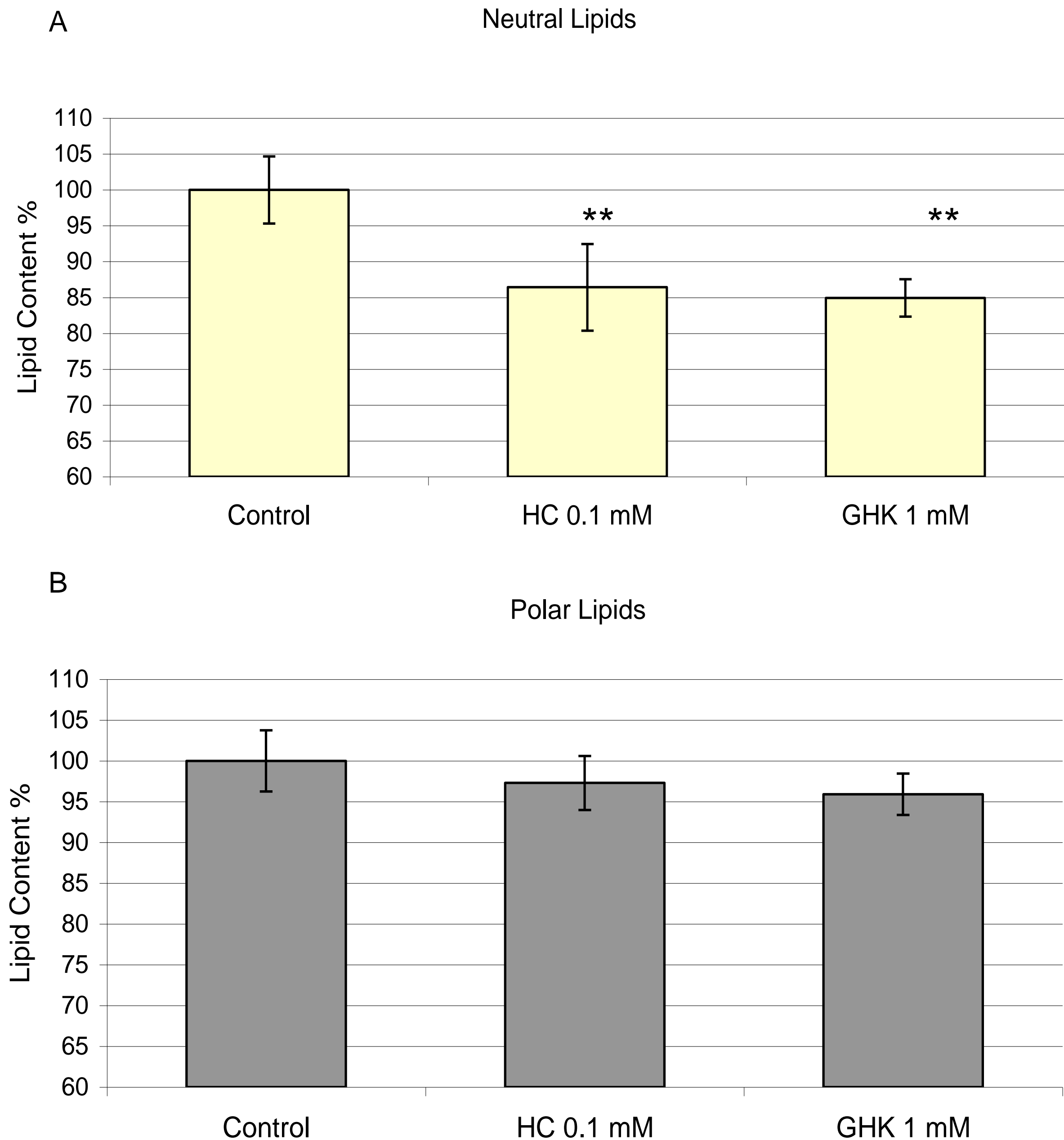


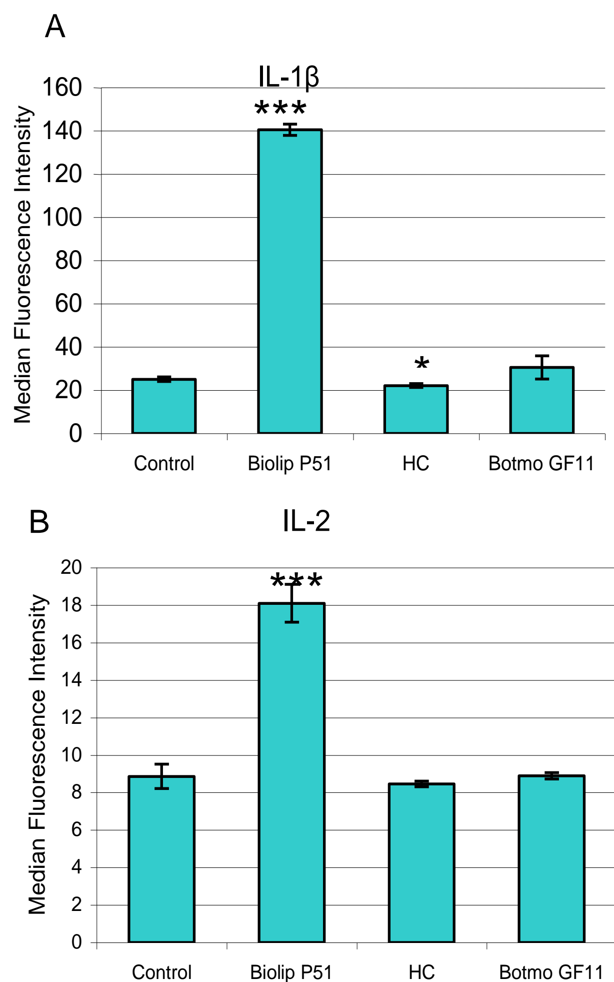
Figure 24 - Lipids in SZ95 sebocytes incubated with glycyl-histidyl-lysine. SZ95 sebocytes were seeded at a density of 40,000 cells/well in a 96-well plate and incubated for 24 h. On the next day cells were pre-treated with arachidonic acid for 1 h, and then treated with GHK in different concentrations. Neutral lipids (yellow bars) and polar lipids (grey bars) were measured with Nile Red staining. Lipid production of control cells with no treatment was set to 100%. Data are shown as mean ± standard deviation of triplicates (n = 9). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

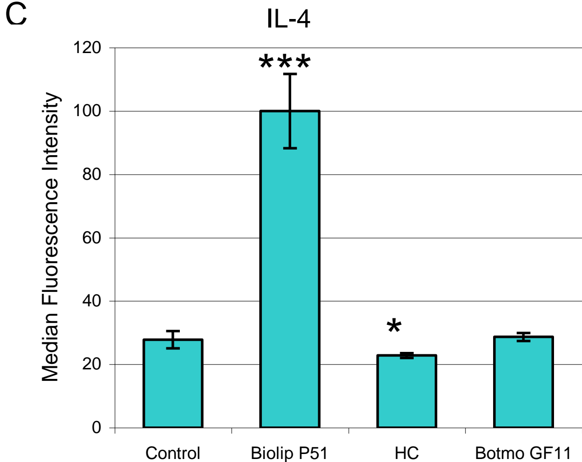
5.4 CYTOKINE ASSAY - BIO PLEX TECHNOLOGY

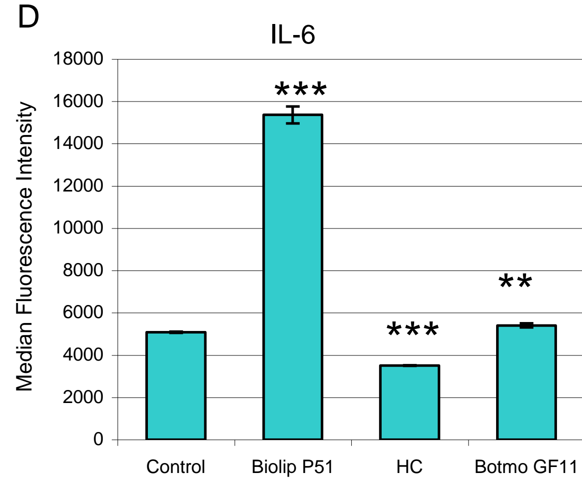
5.4.1 DETECTION OF CYTOKINE LEVELS IN BOTMO GF 11 TREATED SZ95 SEBOCYTES

To identify inflammatory mechanisms, this study determined the release of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, GM-CSF, IFN-γ and TNF-α in treated SZ95 sebocytes. More than 120 beads/well were measured with Bio Plex fluorescence technology (cf. 4.2.9). In the Bio Plex test system median fluorescence intensities (MFI) were measured. From the ratio between MFIs and standard dilution and their concentrations, the concentration of every measurement was derived. Diagrams show the effectively measured values in MFI, whereas the text references give the calculated amounts in pg/ml.

SZ95 sebocytes grown in cell culture medium released IL-6 and IL-8 in higher statistically significant quantities. All other cytokines, IL-1β, IL-2, IL-4, IL-10, IL-12, IL-13, GM-CSF, IFN-γ and TNF-α, were not measured in significantly high amounts.




C
IL-4
Median Fluorescence Intensity
120
100
80
60
40
20
0
***
*
Control
Biolip P51
HC
Botmo GF11


D
IL-6
Median Fluorescence Intensity
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16000
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8000
6000
4000
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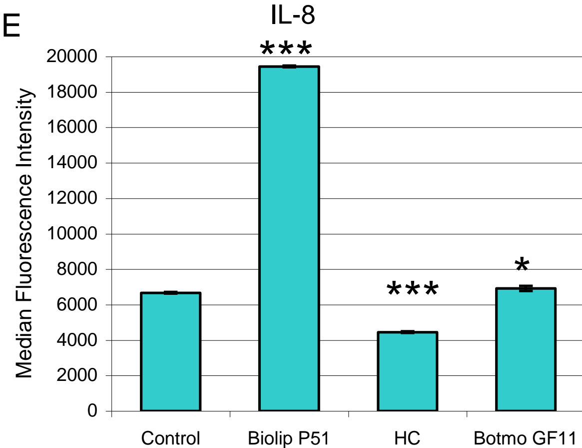
Control
Biolip P51
HC
Botmo GF11


E
IL-8
Median Fluorescence Intensity
20000
18000
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14000
12000
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8000
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4000
2000
0
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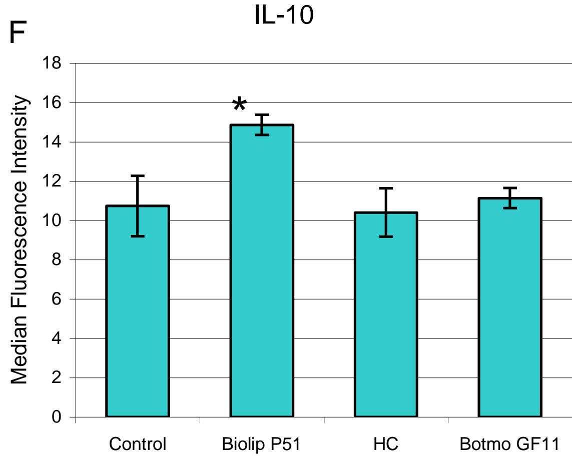
Control
Biolip P51
HC
Botmo GF11


F
IL-10
Median Fluorescence Intensity
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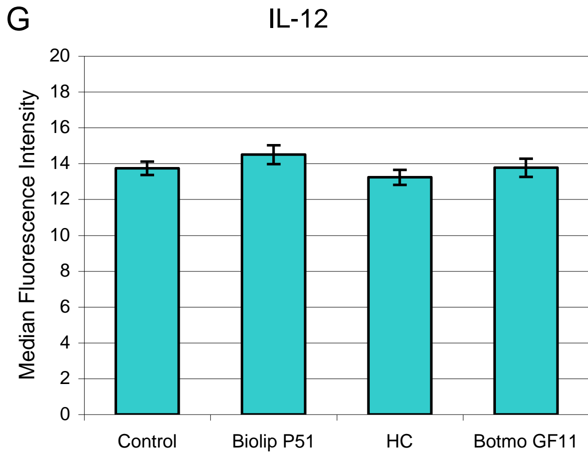
Control
Biolip P51
HC
Botmo GF11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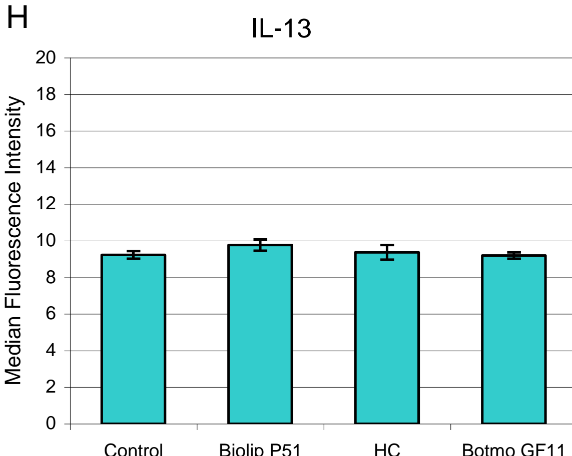
HC
Botmo GF11


H
IL-13
Median Fluorescence Intensity
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Control
Biolip P51
HC
Botmo GF11

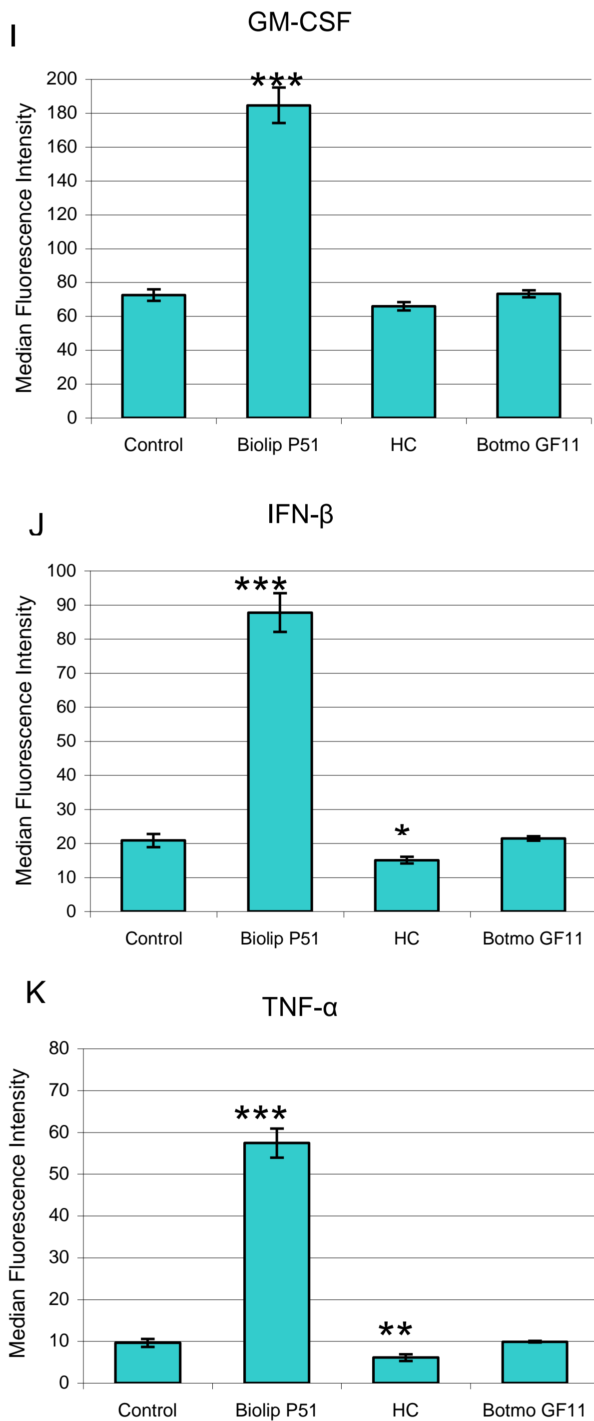


Figure 25 - Cytokine measurements: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, GM-CSF, IFN-γ, and TNF-α. SZ95 sebocytes were seeded at a density of 40,000 cells/well in a 96-well plate and incubated for 24 h. Fluorescence was measured in MFI (Median Fluorescence Intensity). SZ95 sebocytes were treated for 24 h with Biolip P 51 (50 µg/ml), hydrocortisone (HC) (100 µM) and Botmo GF 11 (1 µg/ml). Graph A shows IL-1β cytokine release in SZ95 sebocytes, graph B IL-2, graph C IL-4, graph D IL-6, graph E IL-8, graph F IL-10, graph G IL-12, graph H IL-13, graph I GM-CSF, graph J IFN-γ and graph K TNF-α release in SZ95 sebocytes. Data are shown as mean ± standard deviation of triplicates (n = 3). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

Biolip P 51 supernatant (50 μg/ml) was used as stimulator of proinflammatory cytokines (positive control). Biolip P 51 supernatant induces significantly ($p < 0.001$) IL-1β, IL-2, IL-4, IL-6, IL-8, GM-CSF, IFN-γ, and TNF-α release.
Hydrocortisone (100 μM) was used as negative control, which suppressed significant IL-6 and IL-8 expression ($p < 0.001$). Additionally, IL-1 ($p < 0.05$), IL-4 ($p < 0.05$), IFN-γ ($p < 0.05$) and TNF-α ($p < 0.05$) expression were reduced by hydrocortisone.

Botmo GF 11 treatment stimulated significantly IL-6 ($p < 0.01$) and IL-8 ($p < 0.05$) expression compared to untreated SZ95 sebocytes. Botmo GF 11 treated SZ95 sebocytes showed no significant IL-1β, IL-2, IL-4, IL-10, GM-CSF, IFN-γ and TNF-α cytokine release in comparison to control and untreated cells. No IL-12 and IL-13 release was observed in any SZ95 sebocyte treatment (Figure 25).

5.4.2 CYTOKINE RELEASE IN BOTMO GF 11-101 AND BOTMO GF 11-117 TREATED SZ95 SEBOCYTES

Botmo GF 11-101 (enzymatically inactive) and Botmo GF 11-117 (enzymatically active) were investigated for effect on cytokine release in SZ95 sebocytes. Additionally sebocytes were treated with the enzymatically active snake venom $sPLA_2$ from *Naja mossambica mossambica* (positive control).
Botmo GF 11-101 (1 μg/ml) Botmo GF 11-117 (1 μg/ml) and $sPLA_2$ from *Naja mossambica mossambica* showed no significant IL-6 and IL-8 expression. Treatments induced expression of IL-8 in SZ95 sebocytes, which was higher 10% compared to untreated cells (Figure 26).

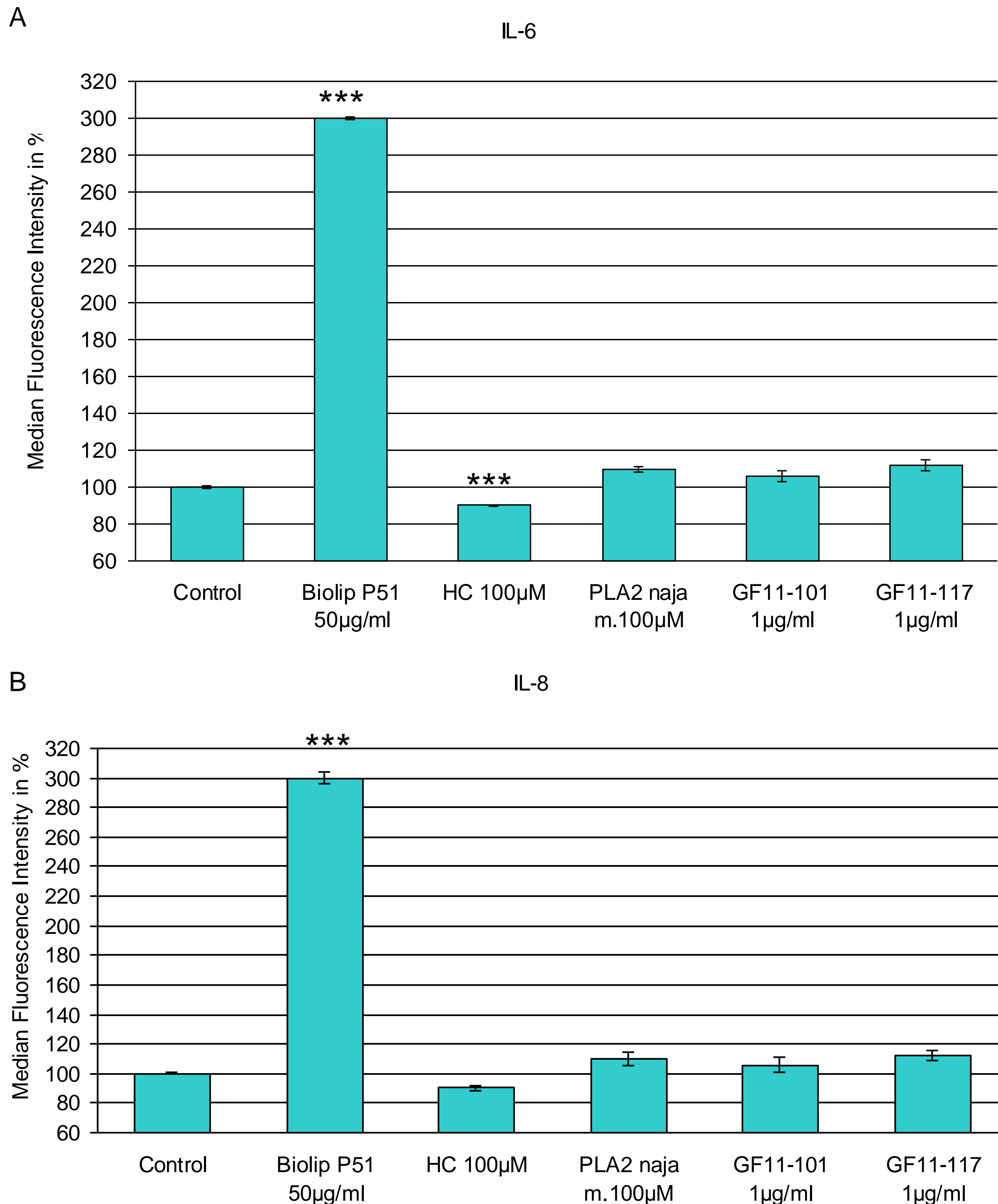


Figure 26 - IL-6 and IL-8 expression in Botmo GF 11-101, Botmo GF 11-117 and sPLA$_2$ *Naja mossambica mossambica* treated SZ95 sebocytes. SZ95 sebocytes were seeded at a density of 40,000 cells/well in a 96-well plate and incubated for 24 h. Fluorescence was measured in Median Fluorescence Intensity. SZ95 sebocytes were treated 24 h with Biolip P 51 (50 µg/ml), hydrocortisone (HC) (100 µM), Botmo GF 11-101 (1 µg/ml), Botmo GF 11-117 (1 µg/ml) and sPLA$_2$ from *Naja mossambica mossambica* venom (PLA$_2$ naja m.) (100 µM). Graph A shows IL-6 cytokine release in SZ95 sebocytes and graph B illustrates IL-8 release in SZ95 sebocytes. Data are shown as mean ± standard deviation of triplicates (n = 6). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

5.4.3 CYTOKINE LEVELS AFTER LIPOXYGENASE INHIBITOR AND CYCLOOXYGENASE-2 INHIBITOR TREATMENT

After treatment of SZ95 sebocytes with general LOX inhibitor (NDGA) and COX-2 inhibitor (NS398) the IL-6 and IL-8 release was measured. In this concentration range no IL-6 or IL-8 expression was measurable in treated SZ95 sebocytes.

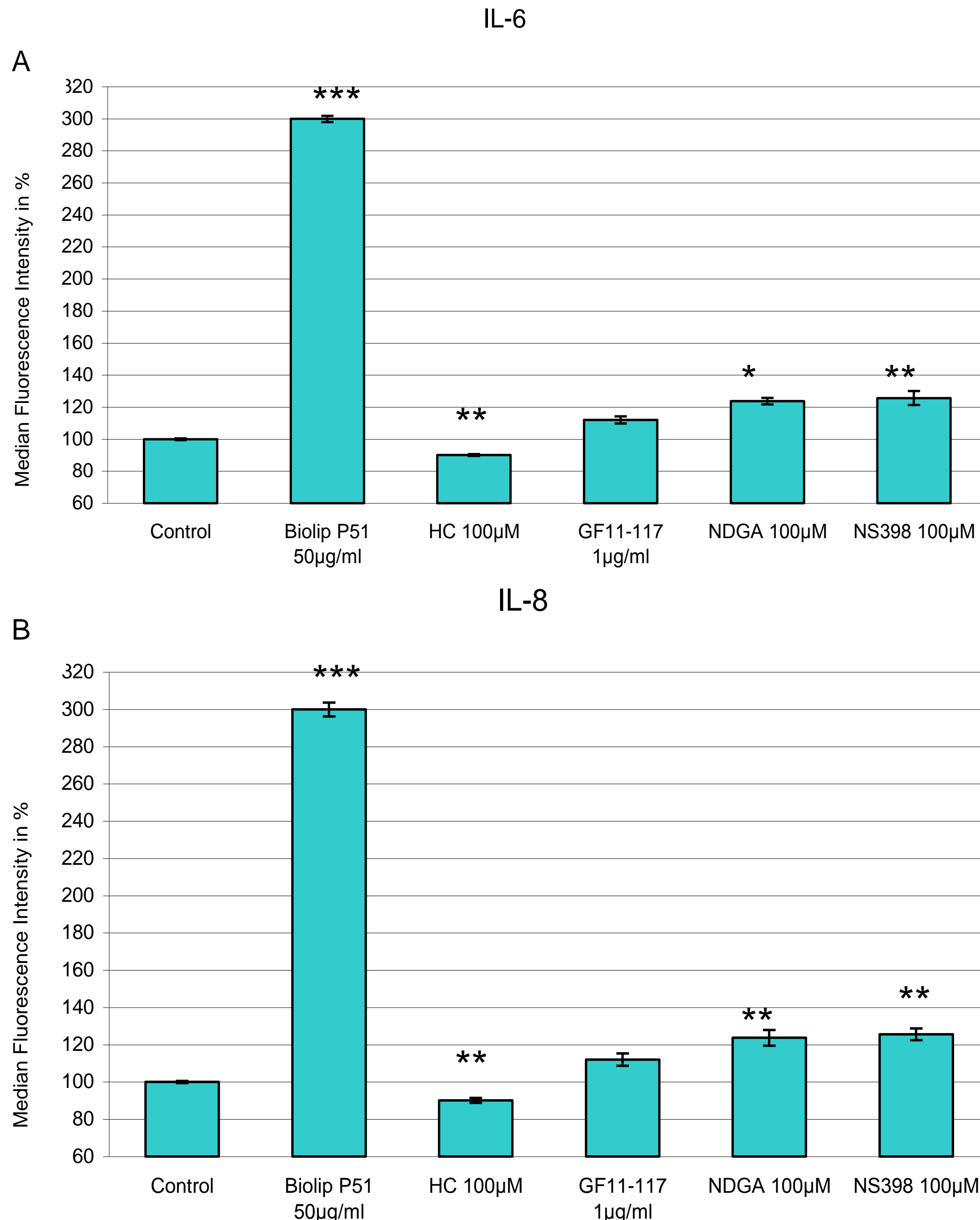


Figure 27 - IL-6 and IL-8 measurement of NDGA and NS398 treated SZ95 sebocytes. SZ95 sebocytes were seeded at a density of 40,000 cells/well in a 96-well plate and incubated for 24 h. Fluorescence was measured in Median Fluorescence Intensity. SZ95 sebocytes were treated for 24 h with Biolip P 51 (50 µg/ml), hydrocortisone (HC) (100 µM), Botmo GF 11-117 (1 µg/ml), NDGA (10 µM) and NS398 (10 µM). Graph A shows IL-6 cytokine release in SZ95 sebocytes and graph B illustrates IL-8 release in SZ95 sebocytes. Data are shown as mean ± standard deviation of triplicates (n = 3). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

5.4.4 5-LIPOXYGENASE AND PHOSPHOLIPASE INHIBITOR SIGNIFICANTLY REDUCED CYTOKINE RELEASE

After having treated SZ95 sebocytes with 5-LOX inhibitor (MK886) and PLA_2 inhibitor ($AACOCF_3$) in similar concentrations to those used in other experiments, both inhibitors showed significant suppression of IL-6 and IL-8 expression.
MK886 reduced significantly IL-6 12% ($p < 0.01$) and IL-8 13% ($p < 0.001$) expression in SZ95 sebocytes. The PLA_2 inhibitor, $AACOCF_3$ inhibited IL-6 12% ($p < 0.01$) expression and decreased IL-8 32% ($p < 0.001$) significantly in SZ95 sebocytes. $AACOCF_3$ was a stronger suppresser of IL-8 expression than MK886 in SZ95 sebocytes

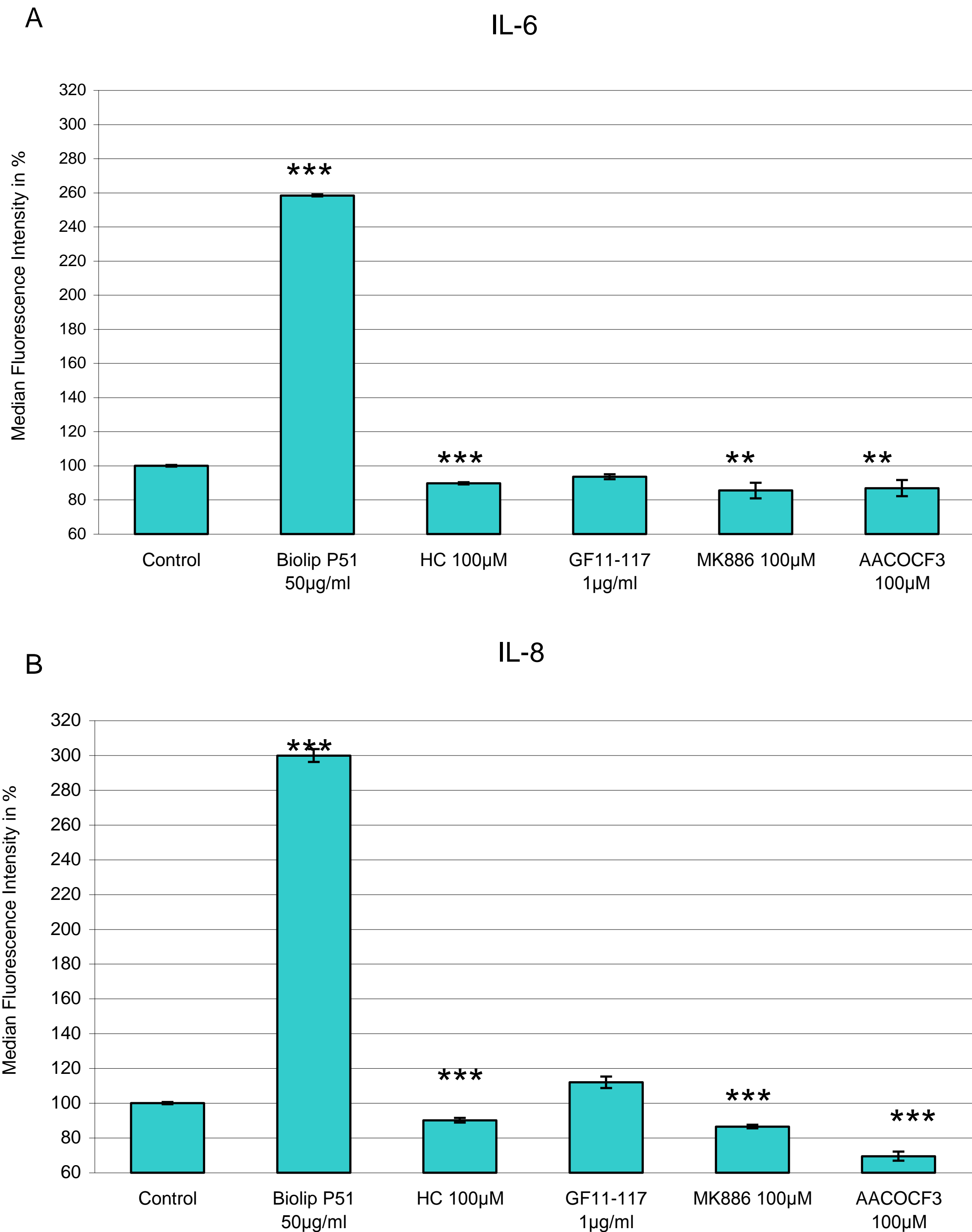
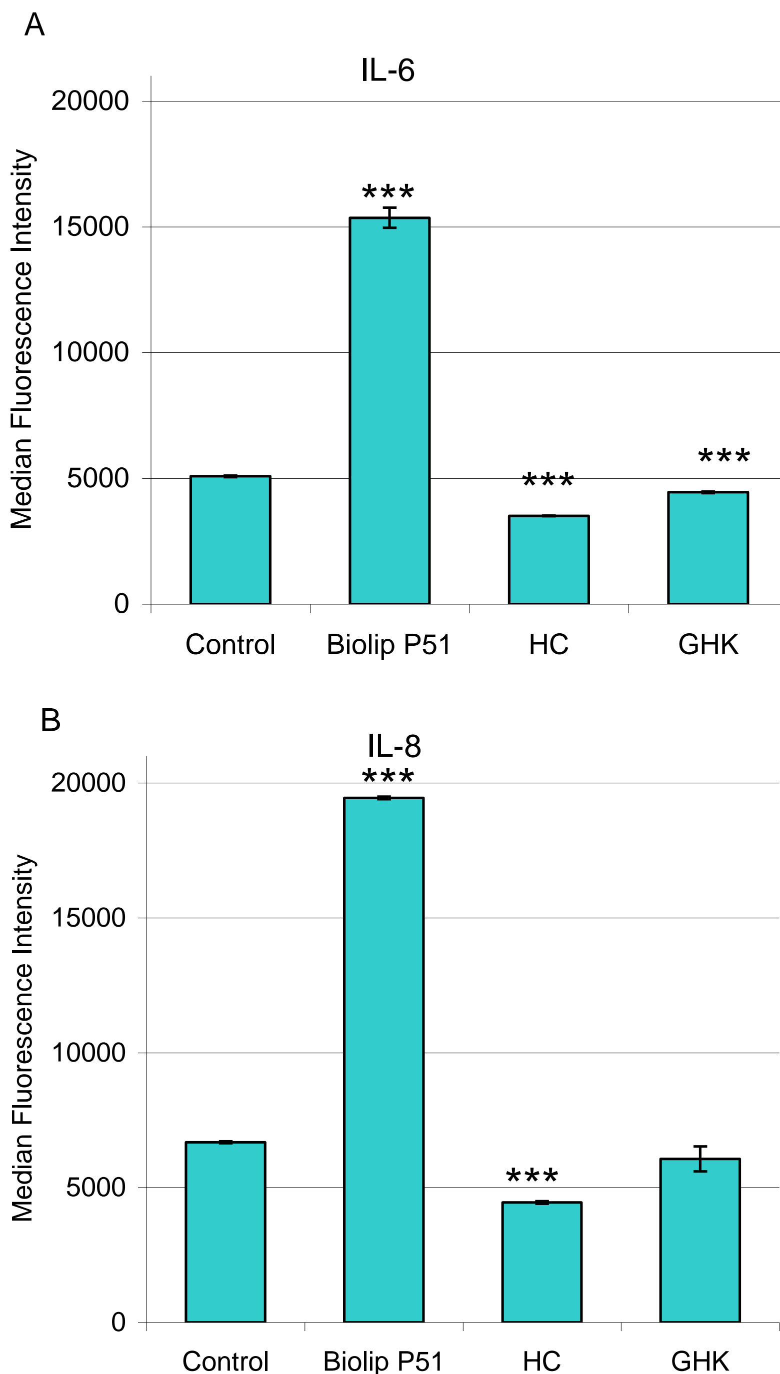


Figure 28 - IL-6 and IL-8 expression in Botmo GF 11-117, MK886 and AACOCF$_3$ treated SZ95 sebocytes. SZ95 sebocytes were seeded at a density of 40,000 cells/well in a 96-well plate and incubated for 24 h. Fluorescence was measured in Median Fluorescence Intensity. SZ95 sebocytes were treated for 24 h with Biolip P 51 (50 µg/ml), hydrocortisone (HC, 100 µM), Botmo GF 11-117 (1 µg/ml), MK886 (10 µM) and AACOCF$_3$ (100 µM). Graph A shows IL-6 cytokine release in SZ95 sebocytes and graph B illustrates IL-8 release in SZ95 sebocytes. Data are shown as mean ± standard deviation of triplicates (n = 3). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

5.4.5 INTERLEUKIN-6 AND INTERLEUKIN-8 RELEASE IN GLYCYL-HISTIDYL-LYSINE TREATED SZ95 SEBOCYTES

Hydrocortisone (0.1 mM) significantly reduced neutral lipid synthesis ($p < 0.01$) in SZ95 sebocytes and had antiinflammatory effects. Figure 29 demonstrates that hydrocortisone treatment decreased IL-6 and IL-8 release ($p < 0.001$) in SZ95 sebocytes. Glycyl-histidyl-lysine (GHK) (1 mM) inhibited IL-6 expression significantly ($p < 0.001$); a low non significant reduction of TNF-α and IL-8 expression could also be shown. At this concentration, GHK reduced IL-6 expression by about 20% and IL-8 by about 14%. All other cytokines, IL-1β, IL-2, IL-4, IL-10, IL-12, IL-13, GM-CSF, IFN-γ and TNF-α were not measured in significant amounts after GHK treatment (data not shown).



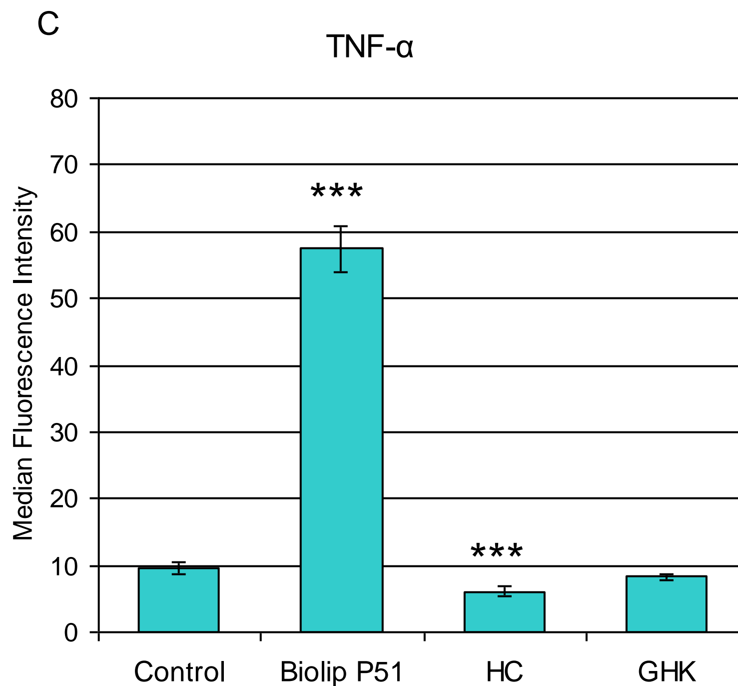


Figure 29 - IL-6 and IL-8 measurements of glycyl-histidyl-lysine on SZ95 sebocytes. SZ95 sebocytes were seeded at a density of 40,000 cells/well in a 96-well plate and incubated for 24 h. Fluorescence was measured in Median Fluorescence Intensity (MFI). SZ95 sebocytes were treated for 24 h with glycyl-histidyl-lysine (GHK) (1 mM), hydrocortisone (HC) (0.1 mM) and Biolip P 51 (50 µg/ml) supernatant. Graph A shows IL-6 cytokine release in SZ95 sebocytes, graph B IL-8 release in SZ95 sebocytes, and graph C TNF-α release in SZ95 sebocytes. Data are shown as mean ± standard deviation of triplicates (n = 3). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

5.5 RECEPTOR BINDING STUDIES – TRANSIENT TRANSFECTION

Bothrops moojeni snake venom contains PLA_2. PLA_2 plays a role in lipid mediator release [47], which may activate PPAR. PPAR receptor binding was investigated by transient transfection methods to identify PLA_2 or PLA_2 metabolites, as PPAR ligands.
For this experiment, COS/7 and SZ95 cells were transfected with PPAR luciferase reporter plasmids specific for PPARα, β, γ_2.

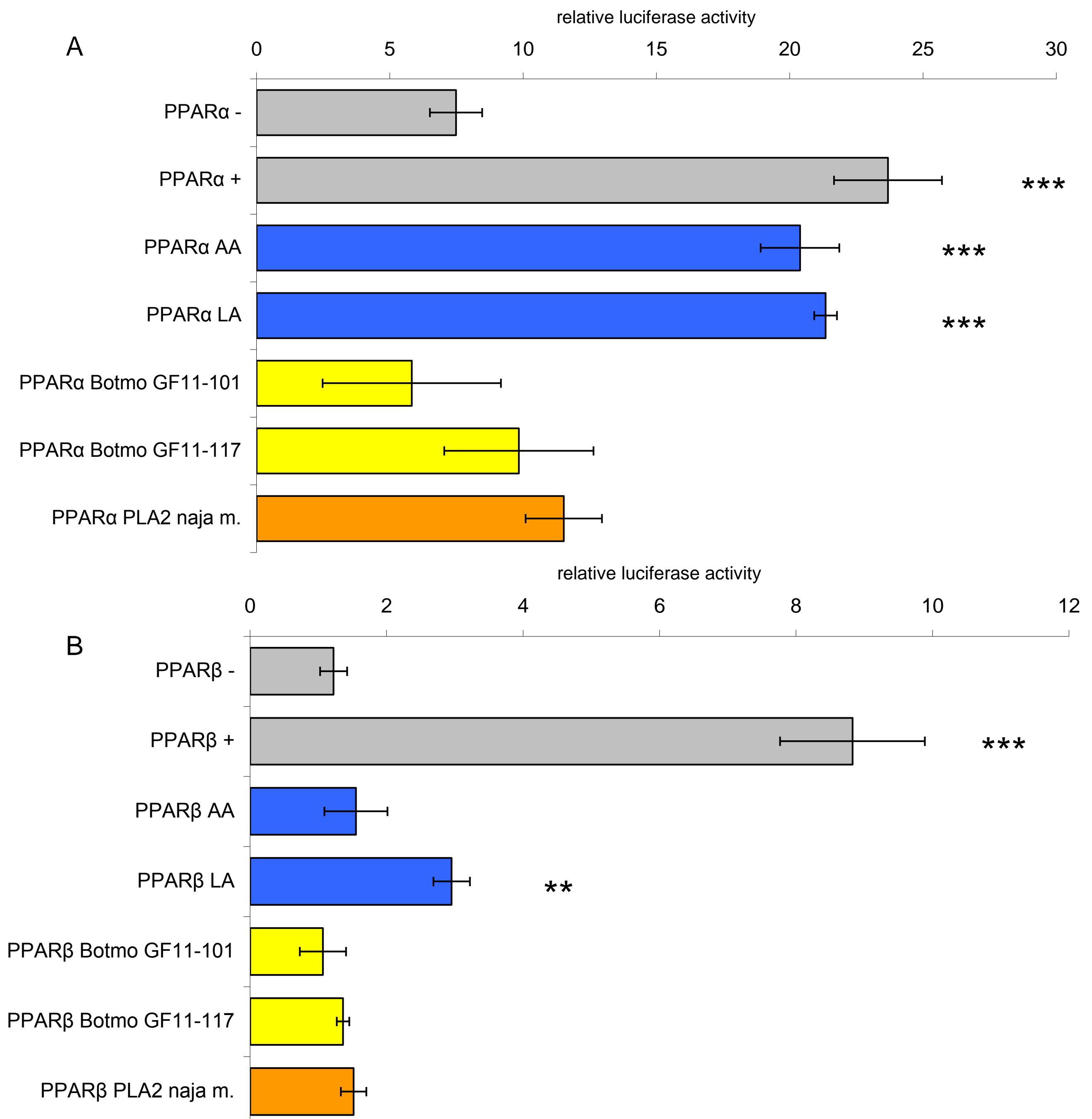
COS/7 cells were used to learn the transient transfection method. Later on, the method was applied on SZ95 sebocytes.

5.5.1 TRANSIENT TRANSFECTION OF COS/7 CELLS

After transfection with promoter-luciferase reporter plasmids, treatments were added in serum free medium as indicated, and cells were incubated for further 24 h. *Firefly* and *Renilla* luciferase activities were measured using the dual luciferase kit. The firefly luciferase values of each sample were normalized by *Renilla* luciferase activity, and data were reported as relative light units.

Promoter-luciferase reporter plasmids, PPAR reporter gene or PPAR response elements (PPAE) were transiently transfected into cells at the same time. This PPAR reporter gene is a gene that encodes an easily assayed product that is coupled to the upstream sequence of another gene and transfected into cells. The reporter gene can then be used to see which factors activate PPAR or PPAE response elements in the upstream region of the gene of interest.

In Figure 30 increasing amounts of PPAR activation were measured in transfected cells with constant quantities of PPARα, β and γ_2 in the presence of the corresponding selective ligands. WY14643 (100 μM) was used as specific PPARα ligand, L165041 (1 μM) as specific PPARβ/δ ligand, and rosiglitazone (5 μM) as specific PPARγ_2 ligand.



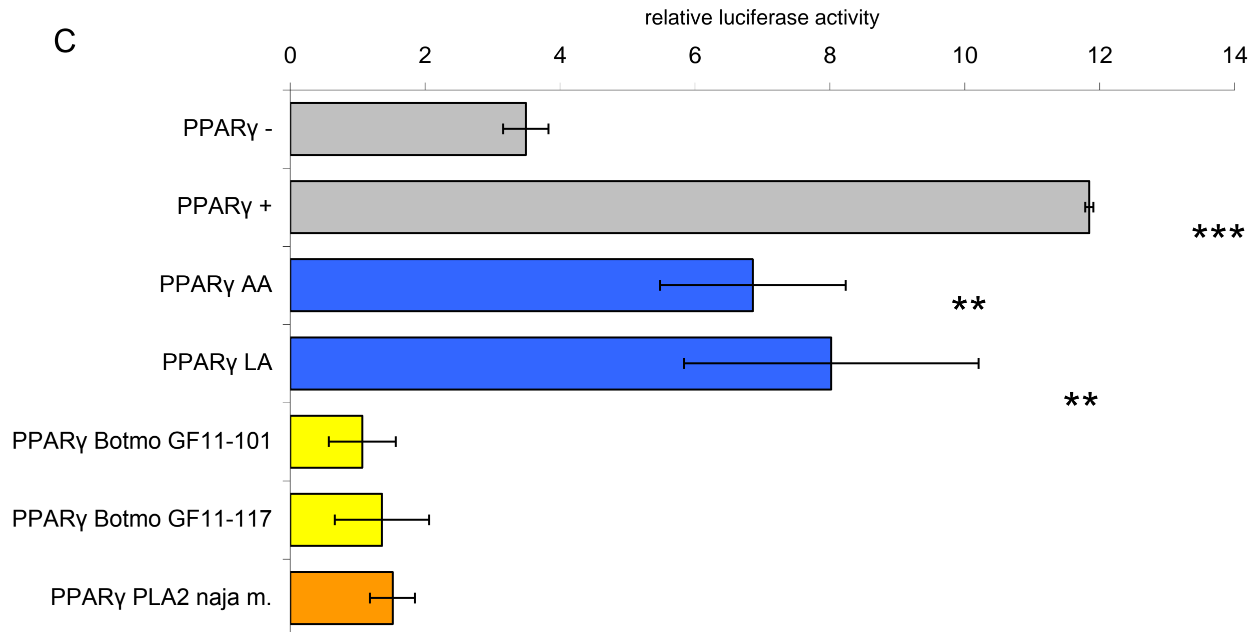


Figure 30 - Identification of PPAR ligands in transiently transfected PPARα, β, γ_2 COS/7 cells. COS/7 cells were seeded at 50 - 60% cell confluency/well in a 12-well plate and incubated overnight. The next day cells were transfected with pSG5 expression vector or PPARα, β, γ_2 and incubated with (-) medium (untreated COS/7 cells), (+) corresponding PPARα, β or γ_2 ligand (WY14643 (100 μM), L165041 (1 μM) and rosiglitazone (5 μM)), arachidonic acid (AA) (50 μM), linoleic acid (LA) (100 μM), Botmo GF 11-101 (1 μg/ml), Botmo GF 11-117 (1 μg/ml) and *Naja mossambica mossambica* $sPLA_2$ (PLA_2 naja m.) (100 μM). Graph A shows COS/7 cells transfected with PPARα, B PPARβ transfected COS/7 cells and C PPARγ_2 transfected Cos/7 cells. Data are shown as mean ± standard deviation of triplicates (n = 6). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

Highest relative luciferase intensity was detected by PPARα transient transfected COS/7 cells. Arachidonic acid and linoleic acid were shown to regulate gene expression through direct and significant interactions with PPARα ($p < 0.001$) and PPARγ_2 ($p < 0.01$). Significant interactions with PPARβ ($p < 0.01$) were observed with linoleic acid treatment.
Botmo GF 11-101 (1 μg/ml) did not activate any PPAR subtype. Botmo GF 11-117 (1 μg/ml) and *Naja mossambica mossambica* $sPLA_2$ (100 μM) were not required for statistically significant PPAR activation in transiently transfected COS/7 cells.

5.5.2 TRANSIENT TRANSFECTION OF SZ95 SEBOCYTES

In SZ95 sebocytes PPARα, β/δ, γ_1, γ_2 expression was detected [19]. This study is based on the hypothesis that lipid synthesis stimulating compounds bind and activate PPAR subtypes directly or indirectly.
Based on the knowledge gained from transient transfection of HeLa cells (data not shown) and COS/7 cells, a new transient transfection method for SZ95 sebocytes was developed. The concentrations of the compounds used for treatment of the cells were similar to those which were used in sebocyte screening. Figure 31 shows the data of a control experiment in order to check if PPAR ligands can activate endogenous PPAR receptors with and without PPAR transient transfection in SZ95

sebocytes. Additionally, these empty vector transiently transfected SZ95 sebocytes were measured and used to ensure that DNA concentrations were constant in each transfection.

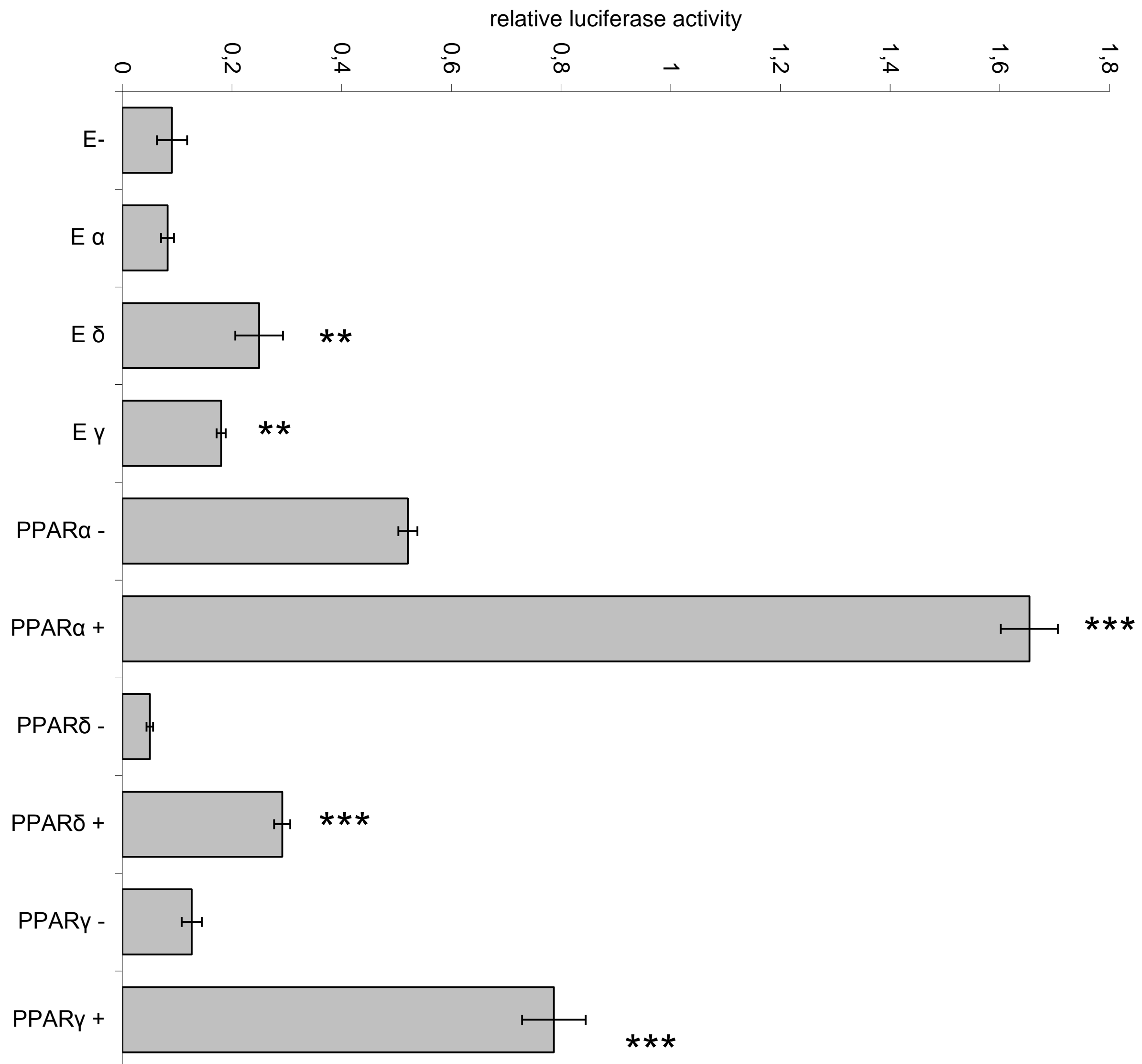


Figure 31 - Relative luciferase activity measurement of transfected and treated SZ95 sebocytes. SZ95 sebocytes were seeded at a density of 200,000 cells/well in a 12-well plate and incubated overnight. On the next day cells were transfected with pSG5 expression vector or PPARα, δ, γ_2 and activated with respective PPARα, δ, γ_2 ligand. (-) medium (untreated SZ95 sebocytes), (+) corresponding PPAR ligand (WY14643 (100 μM), L165041 (1 μM) and rosiglitazone (5 μM)) Data are shown as mean ± standard deviation of triplicates (n = 3). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

E -	PSG5 expression vector transfection and control medium treatment
E α	PSG5 expression vector transfection and PPARα ligand (WY14643) treatment
E δ	PSG5 expression vector transfection and PPARδ ligand (L165041) treatment
E γ	PSG5 expression vector transfection and PPARγ ligand (rosiglitazone) treatment
PPARα -	PPARα transfection and control medium treatment
PPARα +	PPARα transfection and PPARα ligand (WY14643) treatment
PPARδ -	PPARδ transfection and control medium treatment
PPARδ +	PPARδ transfection and PPARβ ligand (L165041) treatment
PPARγ -	PPARγ transfection and control medium treatment
PPARγ +	PPARγ transfection and PPARγ ligand (rosiglitazone) treatment

As seen in Figure 31, SZ95 sebocytes showed an endogenous PPARδ ($p < 0.01$) and PPARγ activity ($p < 0.01$). These results act as negative control for PPARα, δ, γ_2 transiently transfected SZ95 sebocytes.
All three transiently transfected PPAR subtypes were activated significantly by PPARα, δ, γ_2 ligands ($p < 0.001$). SZ95 sebocytes transiently transfected with PPARα without PPAR ligand treatment showed a low luciferase activity. PPARα transfected SZ95 sebocytes treated with PPARα ligand WY14643 (100 μM) led to a significant increase in luciferase activity ($p < 0.001$). Significant PPARδ activation ($p < 0.001$) was observed after treatment with PPARδ ligand L165041 (1 μM). This PPARδ activation was 5 times stronger compared to non treated control cells. The activation was similar to results in pSG5 expression vector (empty vector) transfected cells with L165041 (1 μM) treatment.
A significant activation ($p < 0.001$) with PPARγ ligand rosiglitazone (5 μM) was confirmed (Figure 31).

Additionally, lipid synthesis in SZ95 sebocytes was activated by PPARα ligand (WY14643). WY14643 enhanced neutral lipid synthesis ($p < 0.01$). L165041 and rosiglitazone did not show any effect on the lipid content in SZ95 sebocytes (data not shown).

5.5.3 PPAR ACTIVATION BY FATTY ACIDS AND PHOSPHOLIPASE A_2

As a control, SZ95 sebocytes transfected with the ''empty'' pSG5 vector were treated with arachidonic acid, linoleic acid and compounds like Botmo GF 11-101, Botmo GF 11-117, *honey bee* PLA_2 and *Naja mossambica mossambica* snake venom PLA_2. This experiment determined the effect of the compounds on endogenous PPAR in SZ95 sebocytes. Only linoleic acid significantly increased luciferase activity ($p < 0.05$) in transiently transfected SZ95 sebocytes.

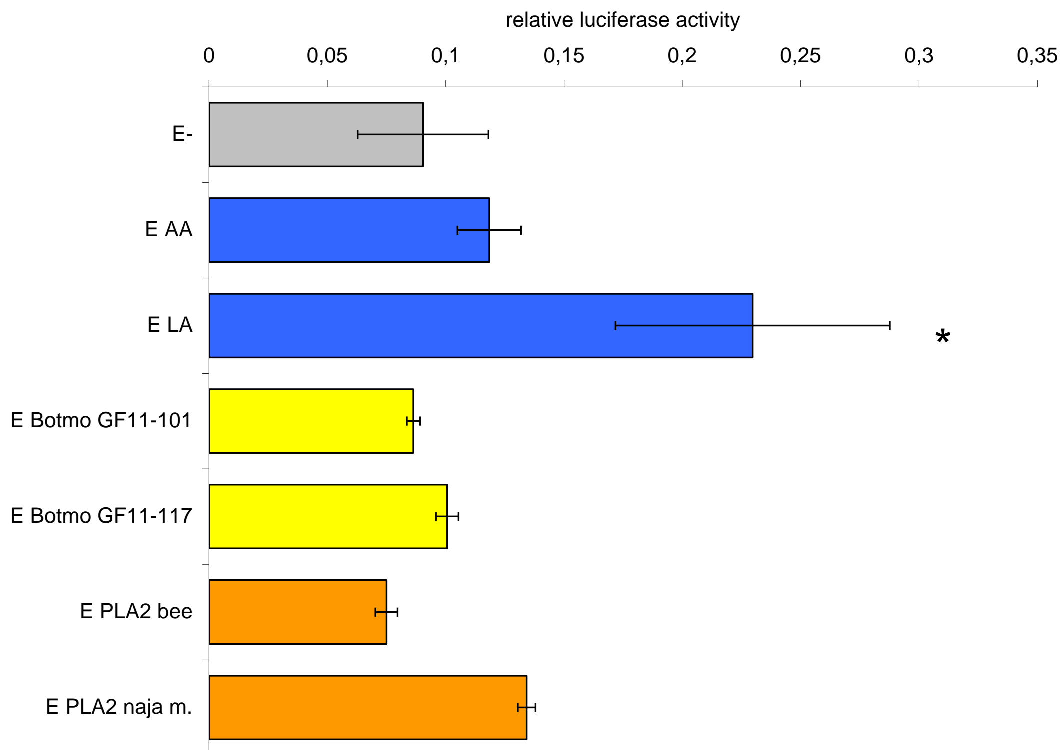
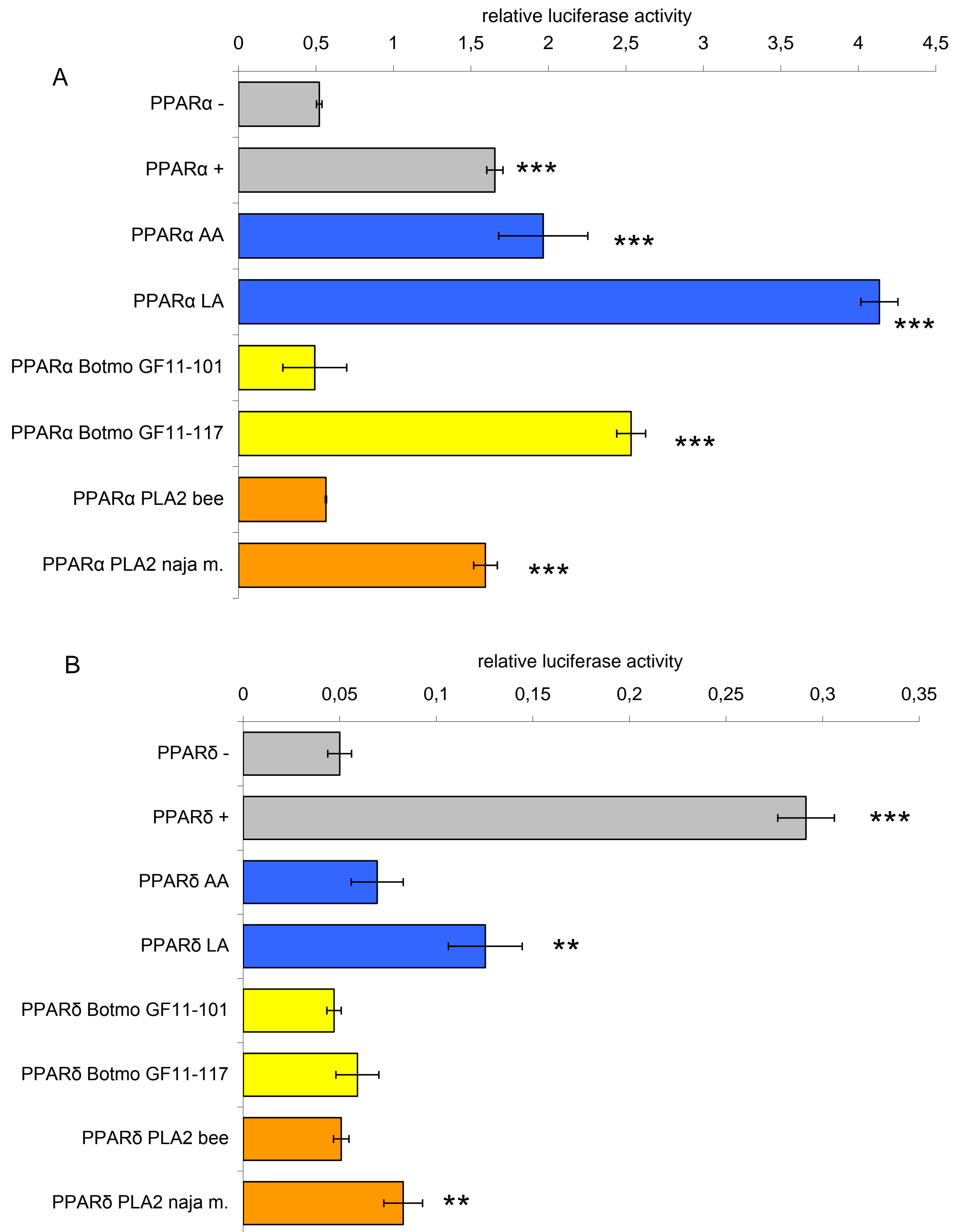


Figure 32 - Identification of endogenous PPAR activation in transiently transfected SZ95 sebocytes.
SZ95 sebocytes were seeded at a density of 200,000 cells/well in a 12-well plate and incubated overnight. On the next day cells were transfected with pSG5 expression vector and incubated with (-) medium (untreated SZ95 sebocytes), arachidonic acid (AA) (50 μM), linoleic acid (LA) (100 μM), Botmo GF 11-101 (1 μg/ml), Botmo GF 11-117 (1 μg/ml), honey bee $sPLA_2$ (PLA_2 bee) (100 μM) and *Naja mossambica mossambica* $sPLA_2$ (PLA_2 naja m.) (100 μM). Data are shown as mean ± standard deviation of triplicates (n = 9). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

SZ95 sebocytes were transiently transfected with PPAR isotypes. Arachidonic acid and linoleic acid, as well as *Naja mossambica mossambica* $sPLA_2$ stimulated an increase in luciferase activity for endogenous PPAR activation. However, only linoleic acid (100 μM) activated endogenous PPAR elements in SZ95 sebocytes significantly ($p < 0.01$) (Figure 32).


A
relative luciferase activity
0
0,5
1
1,5
2
2,5
3
3,5
4
4,5
PPARα -
PPARα +
***
PPARα AA
***
PPARα LA
***
PPARα Botmo GF11-101
PPARα Botmo GF11-117
***
PPARα PLA2 bee
PPARα PLA2 naja m.
***
B
relative luciferase activity
0
0,05
0,1
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0,2
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0,3
0,35
PPARδ -
PPARδ +
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PPARδ AA
PPARδ LA
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PPARδ Botmo GF11-101
PPARδ Botmo GF11-117
PPARδ PLA2 bee
PPARδ PLA2 naja m.
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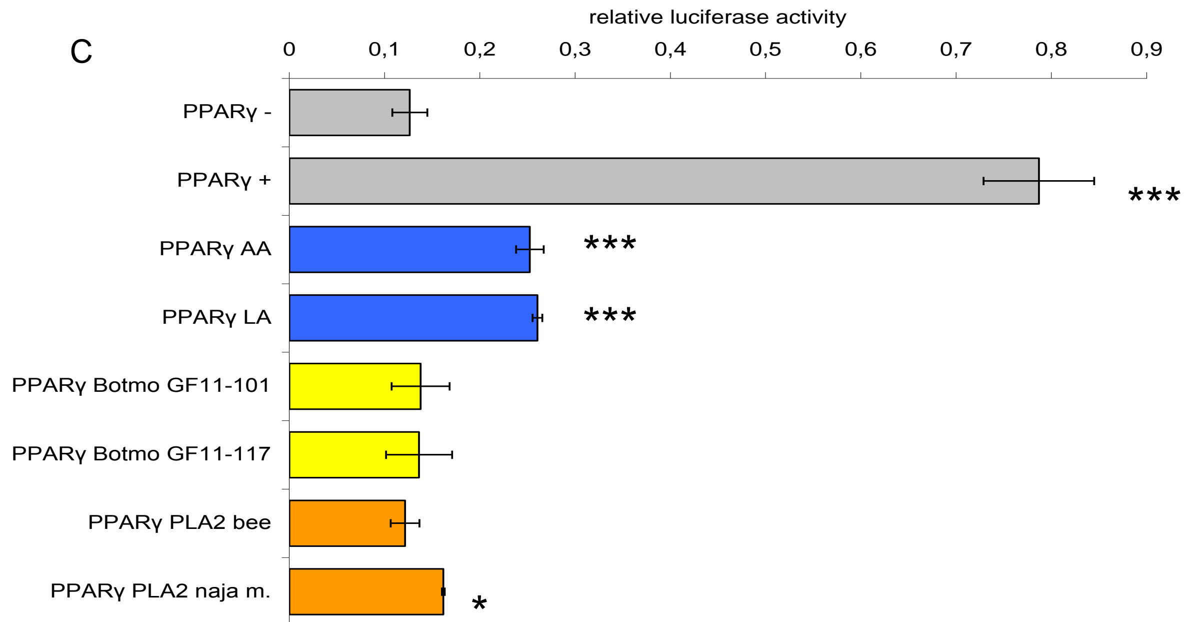


Figure 33 - Identification of ligands for PPARα, δ, γ_2 in transiently transfected sebocytes. SZ95 sebocytes were seeded at a density of 200,000 cells/well in a 12-well plate and incubated overnight. On the next day cells were transfected with PPARα, δ, γ_2 and incubated with (-) medium (untreated SZ95 sebocytes), (+) corresponding PPAR ligand (WY14643 (100 µM), L165041 (1 µM) and rosiglitazone (5 µM)), arachidonic acid (AA) (50 µM), linoleic acid (LA) (100 µM), Botmo GF 11-101 (1 µg/ml), Botmo GF 11-117 (1 µg/ml), *honey bee* $sPLA_2$ (PLA_2 bee) (100 µM) and *Naja mossambica mossambica* $sPLA_2$ (PLA_2 naja m.) (100 µM). Graph A shows SZ95 sebocytes transfected with PPARα, graph B PPARδ transfected SZ95 sebocytes and graph C PPARγ_2 transfected SZ95 sebocytes. Data are shown as mean ± standard deviation of triplicates (n = 6). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

After PPARα activation with PPARα ligands a significant relative luciferase activity ($p < 0.001$) was measured. PPARα activation was 5 to 10 times higher compared to PPARγ_2 or PPARδ.
As shown in Figure 33**,** arachidonic acid, linoleic acid, GF 11-117 and snake $sPLA_2$ significantly induced ($p < 0.001$) PPARα activation in SZ95 sebocytes.
PPARδ was significantly activated by linoleic acid ($p < 0.01$); a low relative luciferase activity was measured with arachidonic acid and snake $sPLA_2$ ($p < 0.01$). A significant activation of PPARγ_2 was observed with arachidonic acid ($p < 0.001$) and linoleic acid ($p < 0.001$); a low significant activation was caused by GF 11-117 ($p < 0.05$). Treatment with Botmo GF 11-101 leads to no PPAR activation. SZ95 sebocytes treated with *Naja mossambica mossambica* $sPLA_2$ activated PPARα with high significance ($p < 0.001$), PPARδ ($p < 0.01$) and PPARγ_2 ($p < 0.05$). In comparison to the snake $sPLA_2$, $sPLA_2$ from *honey bee* venom showed no PPAR activation.

In summary the transient transfection method was for the first time applied and adapted to SZ95 sebocytes. The PPAR reporter gene assay for SZ95 sebocytes was created to determine whether Botmo GF 11-101 or 11-117 could activate PPAR like PPAR ligands. For Hela and COS/7 cells the used transient transfection reagent was lipofectamine. For SZ95 sebocyte transfection, superfect was used instead. Chen et al. reported that sebocytes produce more lipids when they were cultured with medium without fetal bovine serum [19]. Fetal bovine serum inactivates lipofectamine. Additionally, superfect reagent has some more advantages compared to lipofectamine making the transient transfection faster and more reliable.

The PPAR receptor gene assays with SZ95 sebocytes demonstrated PPAR activations with arachidonic acid and linoleic acid treatment. Linoleic acid (100 μM) led to an unspecific activation of all PPAR subtypes. Arachidonic acid (50 μM) only activated PPARα and γ_2. In SZ95 sebocytes only Botmo GF 11-117 and *Naja mossambica mossambica* $sPLA_2$ showed significant PPARα activation (Table 16).

Table 16 - Results overview of transiently transfected SZ95 sebocytes

Treatment	NL	PL	PPARE		PPARα		PPARδ		PPARγ_2	
AA	***	-	137%	-	378%	***	138%	-	260%	***
LA	***	-	267%	*	795%	***	250%	**	205%	***
Botmo GF11-101	**	*	100%	-	94%	-	94%	-	108%	-
Botmo GF11-117	***	*	116%	-	486%	***	107%	-	108%	-
PLA_2 naja m.	***	-	155%	-	306%	***	166%	*	127%	*

Legend: NL neutral lipids, PL polar lipids, (-) no significant effect, AA arachidonic acid (50 μM), LA linoleic acid (100 μM), *Naja mossambica mossambica* $sPLA_2$ (PLA_2 naja m.) (100 μM). PPAR activation is calculated in % compared to PPAR transient transfected sebocytes treated with medium. These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

5.5.4 CYCLOOXYGENASE-2 AND LIPOXYGENASE INHIBITOR TREATMENT OF TRANSFECTED SZ95 SEBOCYTES

Empty vector or PPARα, δ, γ_2 transiently transfected SZ95 sebocytes were treated with arachidonic acid, general LOX inhibitor (NDGA), COX-2 inhibitor (NS398) and in combinations arachidonic acid and NDGA as well as arachidonic acid and NS398.
We started with a control experiment which determined the effect of LOX and COX-2 inhibitors on the empty expression vector transiently transfected SZ95 sebocytes. NDGA as well the combination NDGA with arachidonic acid significantly increased luciferase activity ($p < 0.001$) in expression vector transfected SZ95 sebocytes.
NS398 showed a low, not significant reduction of luciferase activity. Additionally, arachidonic acid and NS398 demonstrated signal suppression compared to sebocytes treatment with arachidonic acid.

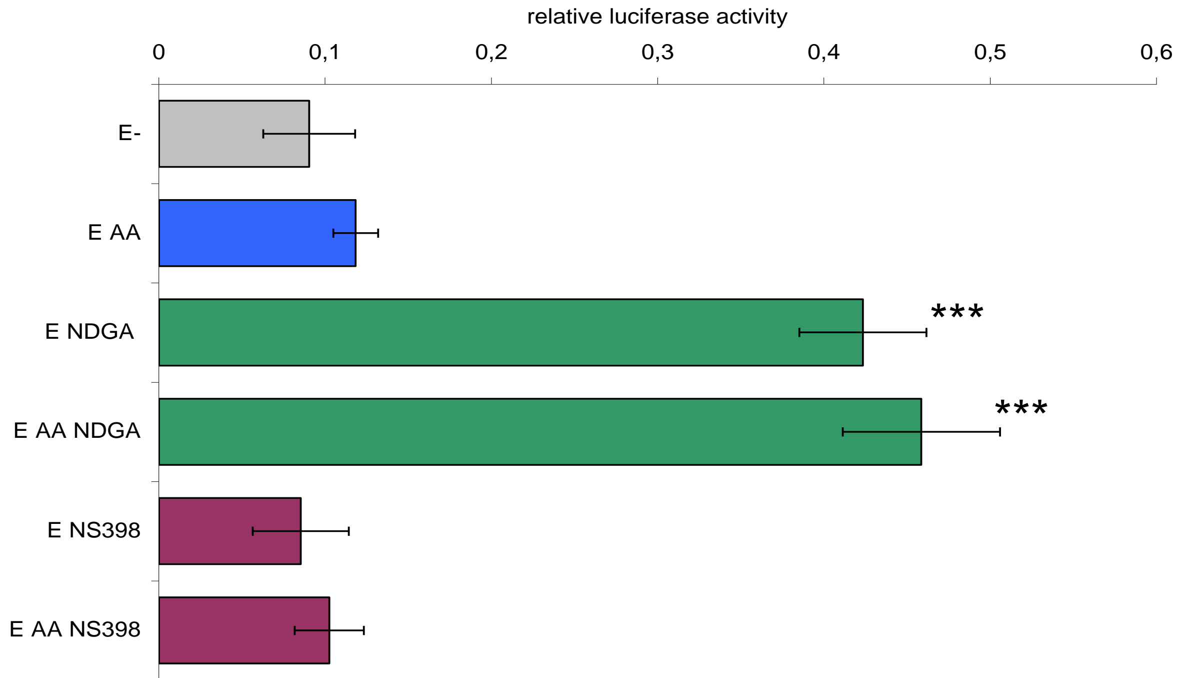
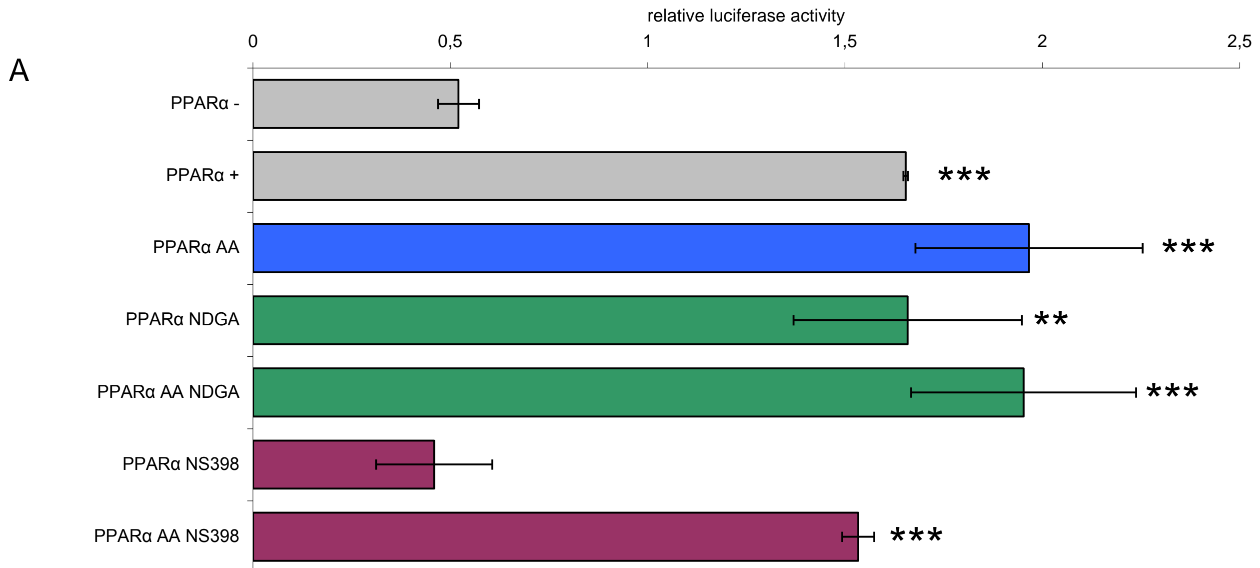


Figure 34 - NDGA and NS398 activation of endogenous PPAR in transiently transfected sebocytes. SZ95 sebocytes were seeded at a density of 200,000 cells/well in a 12-well plate and incubated overnight. On the next day cells were transfected with pSG5 expression vector and incubated with arachidonic acid (AA) (50 µM), NS398 (10 µM) and NDGA (10 µM). Data are shown as mean ± standard deviation of triplicates (n = 3). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

A



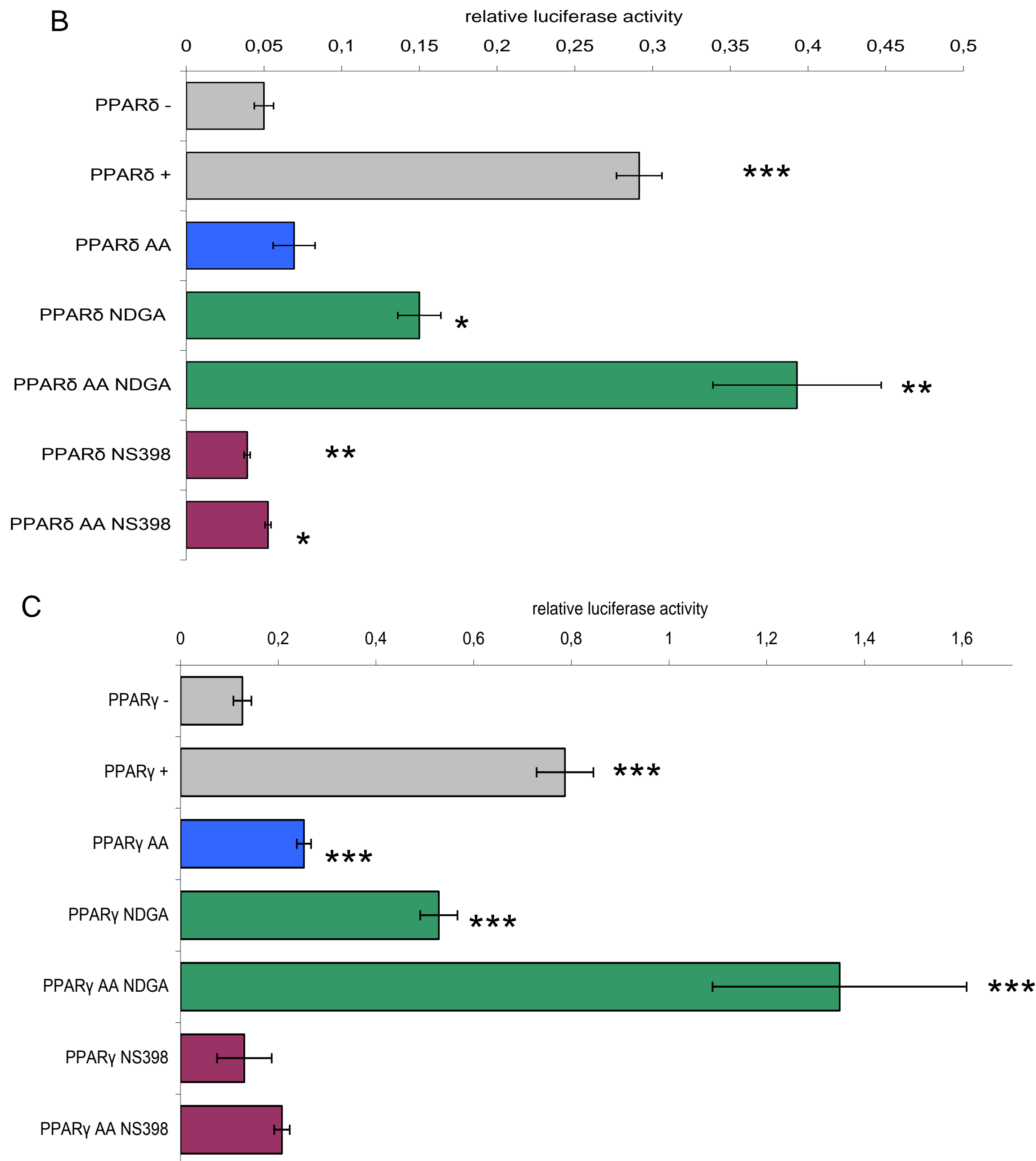


Figure 35 - NDGA and NS398 activation of PPARα, δ, γ_2 in transiently transfected sebocytes. SZ95 sebocytes were seeded at a density of 200,000 cells/well in a 12-well plate and incubated overnight. On the next day cells were transfected with PPARα, δ, γ_2 and incubated with (-) medium (untreated SZ95 sebocytes), (+) corresponding PPARα, δ or γ_2 ligand (WY14643 (100 µM), L165041 (1 µM) and rosiglitazone (5 µM)), arachidonic acid (AA) (50 µM), NS398 (10 µM) and NDGA (10 µM). Graph A shows SZ95 sebocytes transfected with PPARα, graph B PPARδ transfected SZ95 sebocytes, and graph C PPARγ_2 transfected SZ95 sebocytes. Data are shown as mean ± standard deviation of triplicates (n = 3). These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

SZ95 sebocytes were transfected with PPAR luciferase reporter plasmids specific for PPARα, δ, γ_2. After transfection, sebocytes were treated with PPAR ligands, COX-2 inhibitor, NS398 and a general LOX inhibitor, NDGA. It was hypothesized that blocking COX-2 and LOX enzyme activities inhibits PPAR activation by fatty acids and their metabolites.

The known inhibitor of LOX, NDGA, was selected to block general LOX activity in SZ95 sebocytes. Figure 35 shows a highly significant increase of luciferase activity for PPARα activation and a significant increase of PPARδ and γ_2 activation after general LOX inhibitor NDGA treatment. Treatment with arachidonic acid and NDGA demonstrates a synergetic PPARα, δ and γ_2 activation.
Additionally in this sebocyte transfection study, COX-2 inhibitor NS398 was tested for PPAR activation (Figure 35).
In comparison to transfected sebocytes treated with arachidonic acid, combined arachidonic acid and COX-2 inhibitor treated SZ95 sebocytes were able to inhibit PPARα activation. Figure 35 shows still a highly significant PPARα activation and a low PPARγ_2 activation after arachidonic acid and COX-2 inhibitor treatment. NS398 alone shows no activation of PPARα, δ and γ_2.

In summary the LOX inhibitor NDGA activated all PPAR ($p < 0.001$). The combination treatment NDGA and arachidonic acid demonstrated a synergetic, unspecific PPAR activation ($p < 0.001$). However, NDGA did not activate lipid synthesis in SZ95 sebocytes. NS398 exhibited no effect on any PPAR, but NS398 inhibited arachidonic acid activated PPAR (Table 17).

Table 17 - Results overview of cyclooxygenase-2 and lipoxygenase inhibitor treated PPARα, δ, γ_2 transiently transfected SZ95 sebocytes

Result	NL	PL	PPARE		PPARα		PPARδ		PPARγ_2	
AA	***	-	137%	-	378%	***	139%	-	200%	***
NDGA	-	-	490%	***	319%	***	300%	*	418%	***
NDGA+AA	n.d.	n.d.	531%	***	375%	***	787%	**	1067%	***
NS398	-	-	99%	-	88%	-	78%	**	146%	-
NS398+AA	n.d.	n.d.	119%	-	294%	***	105%	*	103%	-

Legend: NL neutral lipids, PL polar lipids, (-) no significant effect, (n.d.) no data, (AA) arachidonic acid (50 µM), NS398 COX-2 inhibitor (10 µM), general LOX inhibitor NDGA (10 µM). PPAR activation is calculated in % compared to PPAR transient transfected SZ95 sebocytes treated with medium. These data were analyzed by two-tailed Student's t test, where ($p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *).

5.6 VISUALISATION AND IDENTIFICATION OF SEBACEOUS LIPIDS

5.6.1 PHASE CONTRAST MICROSCOPE IMAGING OF SZ95 SEBOCYTES

SZ95 sebocytes were treated with linoleic acid, an unspecific neutral PPAR ligand, and arachidonic acid, a neutral PPARα ligand. Both compounds increased neutral lipid accumulation in cells (Figure 36**,** Figure 37**,** Figure 38).
In contrast to untreated sebocytes, cell structure was different and cell size of linoleic acid and arachidonic acid treated SZ95 sebocytes was increased. Furthermore lipid accumulation was stimulated with fatty acid treatment.

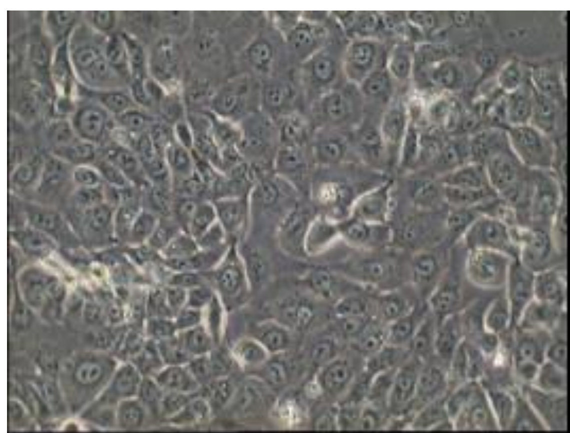


Figure 36 - Cultured SZ95 sebocytes. Cells (20,000 cells/chamber) were seeded in chamber slides and cultured for 48 h (200-times magnified).

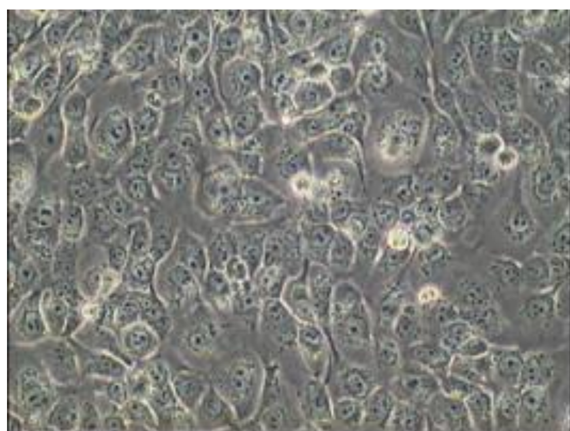


Figure 37 - Cultured SZ95 sebocytes treated with linoleic acid. Cells (20,000 cells/chamber) were seeded in chamber slides and cultured for 24 h. Then SZ95 sebocytes were treated with linoleic acid (0.1 mM) for 24 h. In cytoplasm more lipid droplets were detected (200-times magnified).

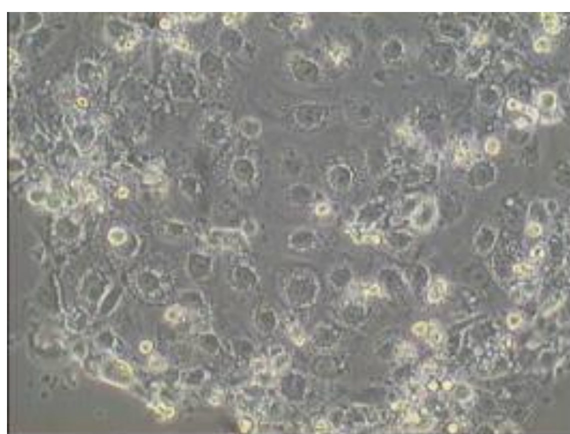


Figure 38 - Cultured, differentiated SZ95 sebocytes treated with arachidonic acid. Cells (20,000 cells/chamber) were seeded in chamber slides and cultured for 24 h. Then SZ95 sebocytes were treated with arachidonic acid ($5x10^{-5}$ M) for 24 h. The cell morphology showed a late differentiation phase. Cell membrane shape became round and showed a lot of lipid droplets inside (200-times magnified).

5.6.2 CONTENT OF LIPIDS IN SZ95 SEBOCYTES INCUBATED WITH LINOLEIC ACID

Cells stained with Nile Red and Hoechst 33342 point out cell structures. This technique allows to visualize accumulated neutral lipids (yellow, Nile Red), membrane lipids (red, Nile Red) and the nucleus (blue, Hoechst). Three images were taken and overlayed (Figure 39).

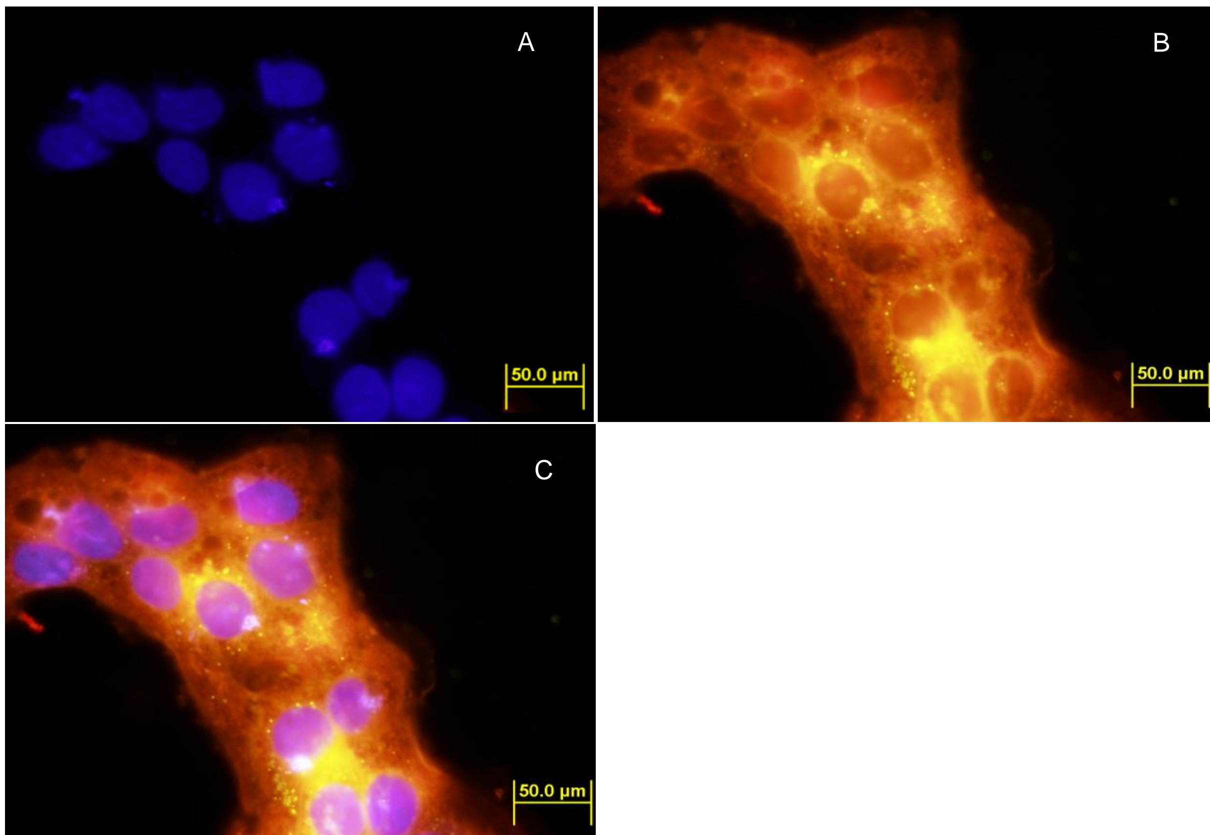


Figure 39 - Fluorescence staining of SZ95 sebocytes. Cells (20,000 cells/chamber) were seeded in chamber slides and cultured for 24 h. Then SZ95 sebocytes were treated with linoleic acid (0.1 mM) for 24 h. Neutral lipids (yellow) (485 / 555 nm), polar lipids (red) (544 / 620 nm) and DNA nucleus (blue) (385 / 420 nm) were visualized by fluorescence microscopy. (A) Staining of the nucleus was done with Hoechst 33342, (B) Nile Red stained polar and neutral lipids, (C) Overlay of picture A and B.

Lipid synthesis in the cells was stimulated with linoleic acid. Many cytoplasmic lipid droplets were on the cell surface, explaining why phase contrast microscopy could not resolve every individual lipid droplet. SZ95 sebocytes were also monitored by confocal microscopy. This method scanned sebocytes in many layers allowing a complete view of the cells and all cytoplasmic droplets. These data were documented in a video sequence. This video showed that SZ95 sebocytes are surrounded by lipid drops (data not shown).

5.6.3 FLUORESCENCE MICROSCOPE IMAGING OF SZ95 SEBOCYTES

Immortalized human SZ95 sebocyte differentiation is characterized by the intracellular accumulation of neutral lipids in the cytoplasm [199]. The lipid synthesis in SZ95 sebocytes was inhibited by hydrocortisone, retinoids and estrogens. It was increased by fatty acids and androgens [191].

5.6.3.1 *VITEX AGNUS CASTUS* EXTRACT INHIBITED LIPID SYNTHESIS

Only fresh *Vitex agnus castus* plant extract was able to inhibit lipid synthesis in SZ95 sebocytes. This extract was not stable and lost its activity after about 3 days at 4°C. This experiment worked only reliably with freshly diluted *Vitex agnus castus* extract.
SZ95 sebocytes were treated with hydrocortisone and *Vitex agnus castus* extract. The morphology of the cells and the cytosolic neutral lipids were documented by means of fluorescence microscopy. In contrast to untreated SZ95 sebocytes, cell size remained unchanged and smaller lipid droplets were visualized in the hydrocortisone and *Vitex agnus casus* retreated cells. However hydrocortisone and *Vitex agnus casus* extract treatment inhibited neutral lipid synthesis (Figure 40) (Figure 41).

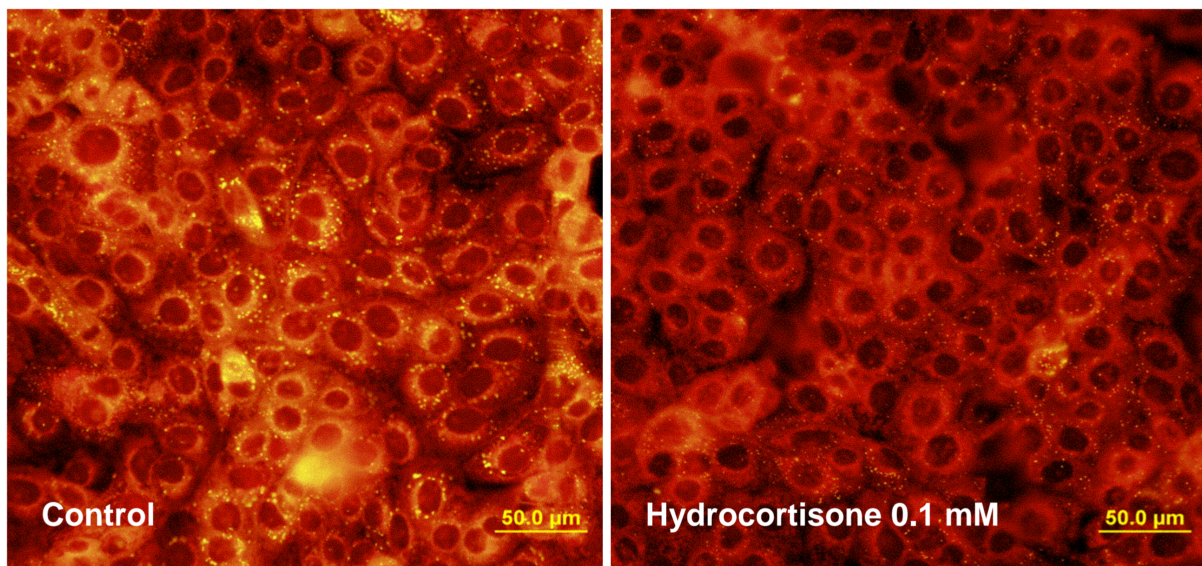


Figure 40 - SZ95 sebocytes under hydrocortisone treatment. SZ95 sebocytes were cultured for 24 h in chamber slides to 100% confluence. One day later SZ95 sebocytes were incubated with hydrocortisone (0.1 mM) for 24 h. Neutral lipids (yellow) (485 / 555 nm) and polar lipids (red) (544 / 620 nm) were visualized by Nile Red fluorescence staining. The control showed the intrinsic lipid production by SZ95 sebocytes which can be inhibited by hydrocortisone.

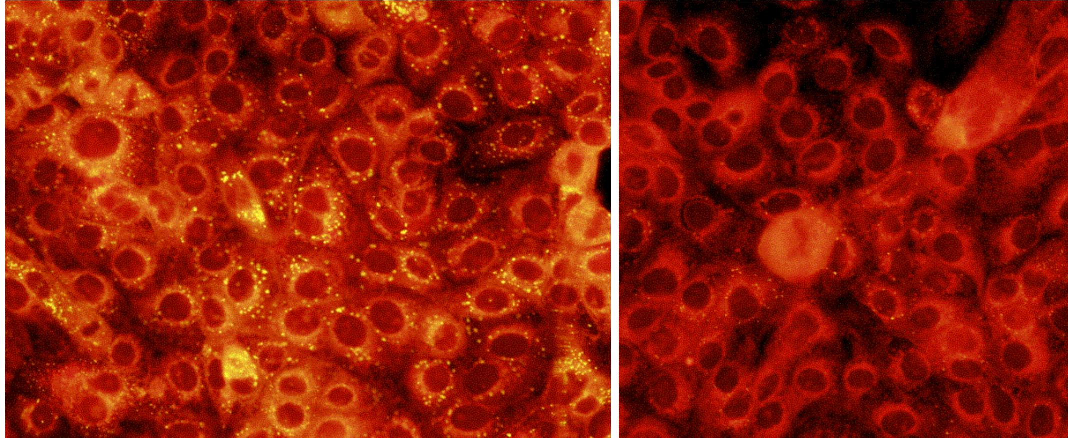


Figure 41 - SZ95 sebocytes under *Vitex agnus castus* water extract treatment. SZ95 sebocytes were cultured for 24 h in chamber slides to 100% confluence. One day later SZ95 sebocytes were incubated with *Vitex agnus castus* water 10 µl/ml extract for 24 h (200-times magnified). Neutral lipids (yellow) (485 / 555 nm) and polar lipids (red) (544 / 620 nm) were visualized by Nile Red fluorescence staining. The control showed the intrinsic lipid production by SZ95 sebocytes which can be inhibited by *Vitex agnus castus* extract.

5.6.3.2 *BOTHROPS MOOJENI* VENOM GEL FILTRATION FRACTION 15 STIMULATED LIPID PRODUCTION

After treatment of SZ95 sebocytes with linoleic acid or Botmo GF 15 for 24 h, morphology of the sebocytes and the neutral and polar lipid synthesis was documented by means of fluorescence microscopy. In comparison with untreated SZ95 sebocytes, cell sizes of treated SZ95 sebocytes seemed to be increased. Furthermore, lipid accumulation was enhanced in linoleic acid and Botmo GF 15 treated cells (Figure 42) (Figure 43).

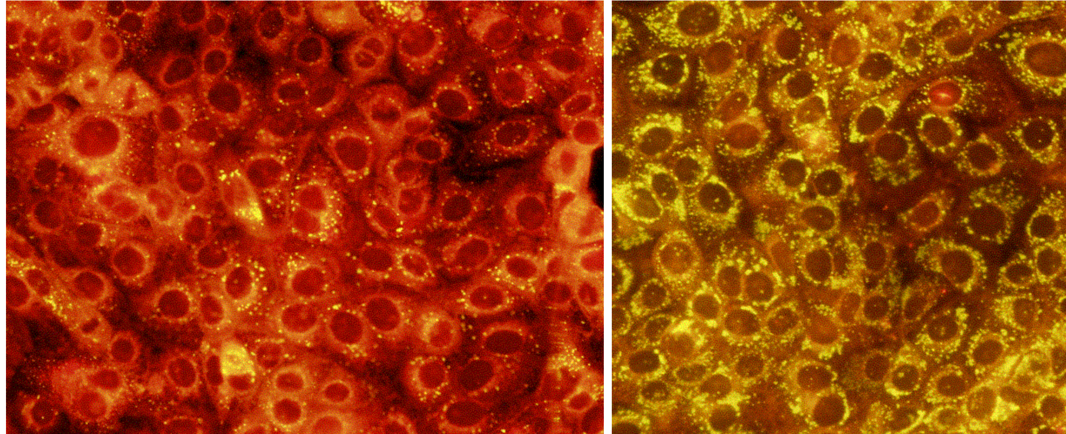


Figure 42 - SZ95 sebocytes under linoleic acid treatment. SZ95 sebocytes were cultured for 24 h in chamber slides to 50% confluence. One day later SZ95 sebocytes were incubated with linoleic acid (0.1 mM) for 24 h (200-times magnified). Neutral lipids (yellow) (485 / 555 nm) and polar lipids (red) (544 / 620 nm) were visualized by Nile Red fluorescence staining. The control showed the intrinsic lipid production by SZ95 sebocytes which can be additionally stimulated by linoleic acid treatment.

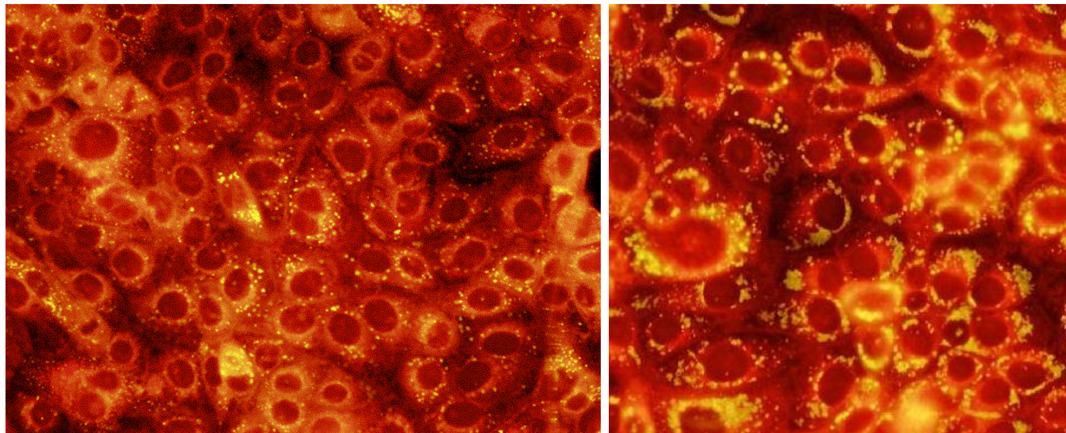


Figure 43 - SZ95 sebocytes under Botmo gel filtration fraction 15 treatment. Cells (20,000 cells/chamber) were seeded in chamber slides and cultured for 24 h. Then SZ95 sebocytes were treated with Botmo GF 15, 1 μg/ml for 24 h (200-times magnified). Neutral lipids (yellow) (485 / 555 nm) and polar lipids (red) (544 / 620 nm) were visualized by Nile Red fluorescence staining. Control cells showed the intrinsic lipid production by SZ95 sebocytes which can be additionally stimulated by Botmo GF15 treatment.

6 DISCUSSION

The normal function of sebaceous glands is to produce and secrete sebum, a mixture of cell debris and lipids including triglycerides, fatty acids, wax esters, squalene, cholesterol esters, and cholesterol [31, 106, 126, 161]. Sebum lubricates the skin to protect the skin surface against outside influences and dehydration.
The sebaceous glands consist of sebaceous gland cells, also called sebocytes. Sebocytes differentiate from the outer margin to the center of the sebaceous gland, increasing their size and lipid content until they burst and die. With advancing age the size of differentiating sebocytes tends to decrease, while their number remains approximately the same throughout life [190]. Sebocytes show an age-related reduced secretory output, which results in a decrease in the surface lipid levels [34, 122] – which is one of the typical signs of aged skin [89].

6.1 APPROACH TO IDENTIFY NEW ACTIVE COMPOUNDS INTERFERING WITH LIPID SYNTHESIS

For this thesis a SZ95 sebocyte screening model was developed to search for new active compounds as described in chapter 4.2.3. The aim was to identify new lipid regulating compounds and gain more insight into regulation of lipid synthesis. Neutral and polar lipids were measured by direct staining of SZ95 sebocytes with Nile Red. At the same time cell viability was monitored by fluorescein diacetate staining. This screening method allows the measurement of multiple parameters in a single well. Two hundred samples from plant extracts, snake venom fractions, microorganism extracts, and synthetic peptides were screened in two different concentrations. Plants and peptides were chosen based on literature research. Criteria for the literature research were plants known in traditional medicine and peptides involved in wound healing, hormone regulation, and lipid regulation. Microorganism samples (Marine microbial mats) came from French Polynesian islands. Until now, there are no data available which show how marine microbial mats act on human skin cells. These substances have to be identified and tested for their pharmaceutical activity.
The size exclusion chromatography of *Bothrops moojeni* crude venom resulted in 18 protein fractions named Botmo GF fraction 1 - 18 (Figure 44) [115]. The potential use of *Bothrops moojeni* venom may be interesting for pharmaceutical effects [74].

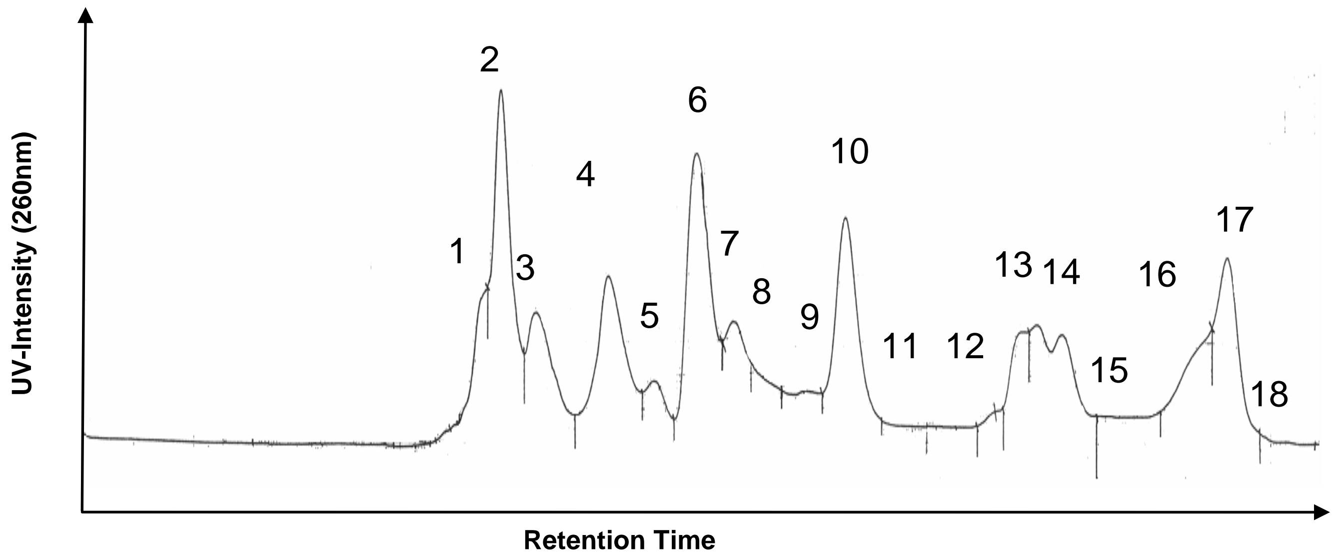


Figure 44 - Gel filtration chromatogram of crude *Bothrops moojeni* venom. Size exclusion chromatography of *Bothrops moojeni* crude venom performed on a Superdex 75 column resulted in 18 fractions named Botmo GF fractions 1-18 (cf. chapter 4.2.1.4) [115, 116].

After SZ95 sebocyte screening 26 compounds showed significant lipid regulating effects either up- or down-regulating of neutral lipids in SZ95 sebocytes. Out of 26 compounds, fractions, extracts, and peptides, which showed interesting results, the *Bothrops moojeni* snake venom gel filtration fractions (Botmo GF) were selected for further evaluation. Botmo GF stimulated highly significantly neutral lipid synthesis in SZ95 sebocytes without cytotoxicity or apoptotic effects (Figure 16).
Other elements of this work were experiments with glycyl-histidyl-lysine derivatives and the *Vitex agnus castus* extract which demonstrated significant neutral lipid suppression. These investigations were only a side project in this work and are discussed in the appendix.

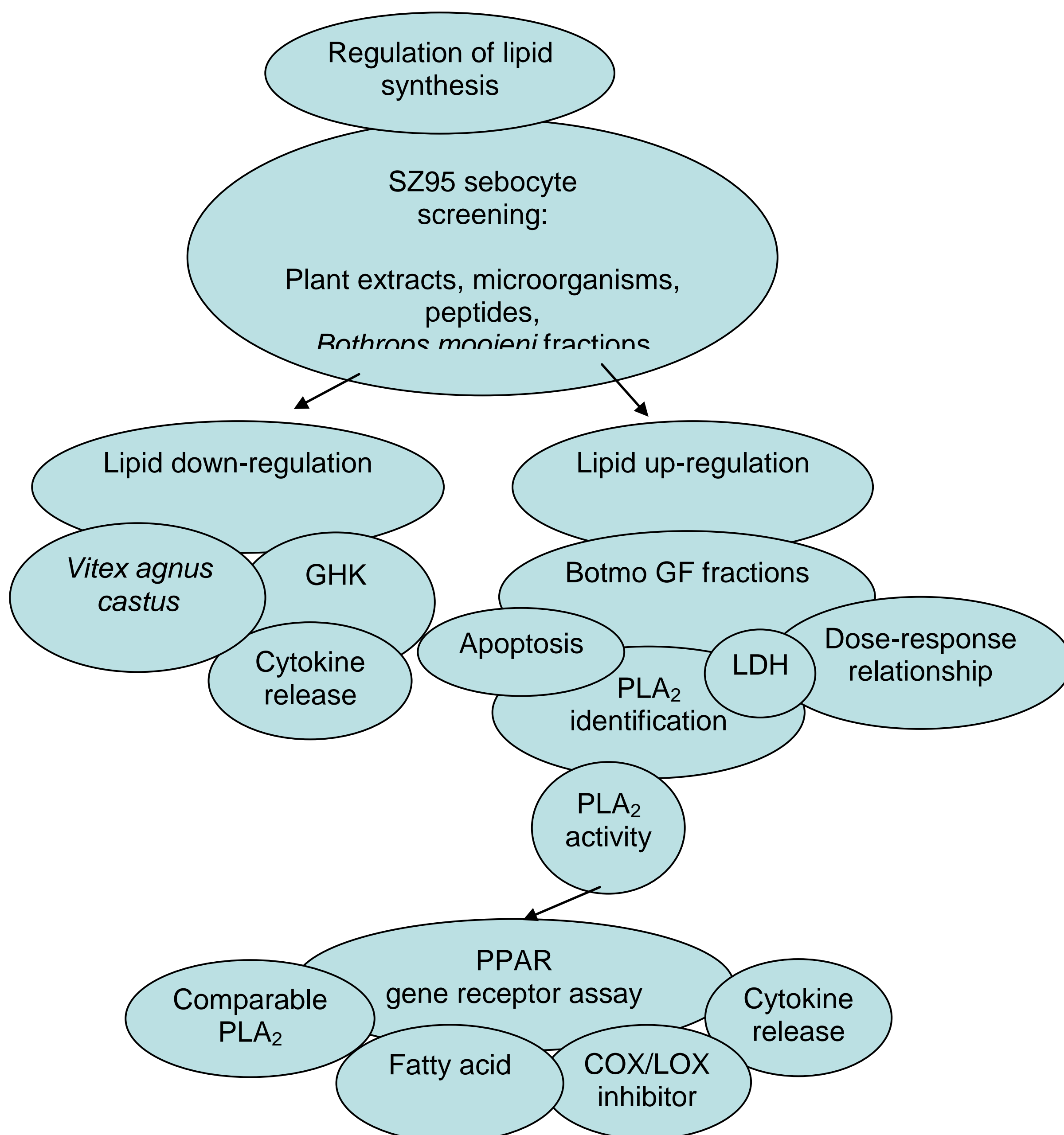


Figure 45 - Focal points to identify new active compounds. This matrix provides a view into the proceedings of this work. Legend: Glycyl-histidyl-lysine (GHK), Lactatedehydrogenase (LDH), Peroxisome proliferator-activated receptors (PPAR), Phospholipase A_2 (PLA_2), Cyclooxygenase (COX), Lipoxygenase (LOX).

Figure 45 displays the approach of this work. With this SZ95 sebocyte screening model, new compounds which regulate neutral and polar lipid synthesis in SZ95 sebocytes were identified. For this work *Bothrops moojeni* venom gel filtration fractions were chosen for up-regulation and *Vitex agnus castus* as well as GHK and GHK derivatives for down-regulation of neutral lipid synthesis in SZ95 sebocytes.
As mentioned before, Botmo GF fractions stimulated lipid production in SZ95 sebocytes and exhibited no apoptotic and cytotoxic effects in the concentrations applied. Additionally cytokine release by Botmo GF fractions treated SZ95 sebocytes was examined, which was statistically not significantly different than the untreated cells.

After identification of $sPLA_2$ type IIA in Botmo GF, it was suggested that PLA_2 could activate the arachidonic acid metabolism in SZ95 sebocytes. Products of the arachidonic acid metabolism like arachidonic acid and linoleic acid increase the lipid production in sebocytes. Both fatty acids are natural PPAR ligands [4, 129, 132, 191].

In order to investigate how Botmo GF fractions stimulated sebocyte lipids, a new PPAR transient transfection method for SZ95 sebocytes was developed. The result of $sPLA_2$ activation may increase substrates for cyclooxygenase (COX), lipoxygenase (LOX) as well as for platelet-activating factor (PAF) pathway (Figure 46) [33]. In this model, Botmo GF fractions, comparable $sPLA_2$, arachidonic acid, linoleic acid, COX-2 and LOX inhibitors were tested for activating PPAR in SZ95 sebocytes. To obtain more information about the lipid signaling pathway, COX and LOX inhibitors were used to block PPAR activation in arachidonic acid activated, PPAR transiently transfected SZ95 sebocytes. Furthermore, COX-, LOX- and PLA_2 inhibitors were tested on how they act on SZ95 sebocyte lipid content or cytokine expression. Their biological actions may be interesting for the investigation of lipogenesis and lipolysis, which may be essential for therapeutic agents for both dry and oily skin.

6.1.1 STIMULATION OF LIPID SYNTHESIS BY *BOTHROPS MOOJENI* PROTEIN FRACTIONS

In this work *Bothrops moojeni* snake venom showed lipid regulatory actions in SZ95 sebocytes. *Bothrops moojeni* snake venom was mostly evaluated for inhibiting blood coagulation [74]. This was the first time *Bothrops moojeni* protein fractions were tested on SZ95 sebocytes. Surprisingly, these water-soluble fractions significantly increased neutral lipid synthesis in SZ95 sebocytes.
Increased cell volume and accumulation of lipid droplets in the cytoplasm are phenomena indicating terminal differentiation for sebocytes. This differentiation state indicates holocrine secretion and cell death [173]. Interestingly, no toxic and apoptotic effects were observed in SZ95 sebocytes with the applied compound concentrations (Figure 16).
All tested Botmo GF fractions 1 - 18 (Figure 44; Table 14) led to the activation of lipid synthesis in SZ95 sebocytes. Botmo GF fractions were further purified using RP-HPLC and analyzed by diverse transversal techniques [115]. The resulting HPLC chromatogram showed that Botmo GF fraction still contained a lot of compounds which were again tested for lipid activation in SZ95 sebocytes. MALDI-TOF MS analyses revealed in the HPLC subfractions the presence of many compounds in the PLA_2 mass range, while immunometric assay for detection of type IIA $sPLA_2$ allowed for characterization of enzymatically active and non-active members of the PLA_2 family [135].
Botmo GF 15-81 to 84 as well as Botmo GF 11-101 and Botmo GF 11-117 increased neutral lipid production in SZ95 sebocytes (Figure 18 and Figure 20). Because of the amount of material and the measured lipid synthesis activation, two Botmo GF 11 PLA_2-containing subfractions were selected for further evaluation of their potential on the stimulation of lipogenesis in SZ95 sebocytes. These were Botmo GF fraction 11-101 and 11-117, deprived of PLA_2 enzymatic activity or possessing it, respectively. HPLC fractions Botmo GF 11-101 (150%) and Botmo GF 11-117 (310%) stimulated significantly neutral lipid production in SZ95 sebocytes (Figure 20).

These findings on enzymatically active or inactive PLA_2, which were detected in the low molecular weight fractions of *Bothrops moojeni* crude venom, are supported by several previous studies that identified many PLA_2 in this snake venom [52, 115, 116].
PLA_2 catalyses the hydrolysis of the sn-2 fatty acyl bond of phospholipids yielding a free fatty acid and a lysophospholipid [22]. The interest in PLA_2 arose due to their unusual adaption for catalysis at the surface between aqueous and lipid phases [142].

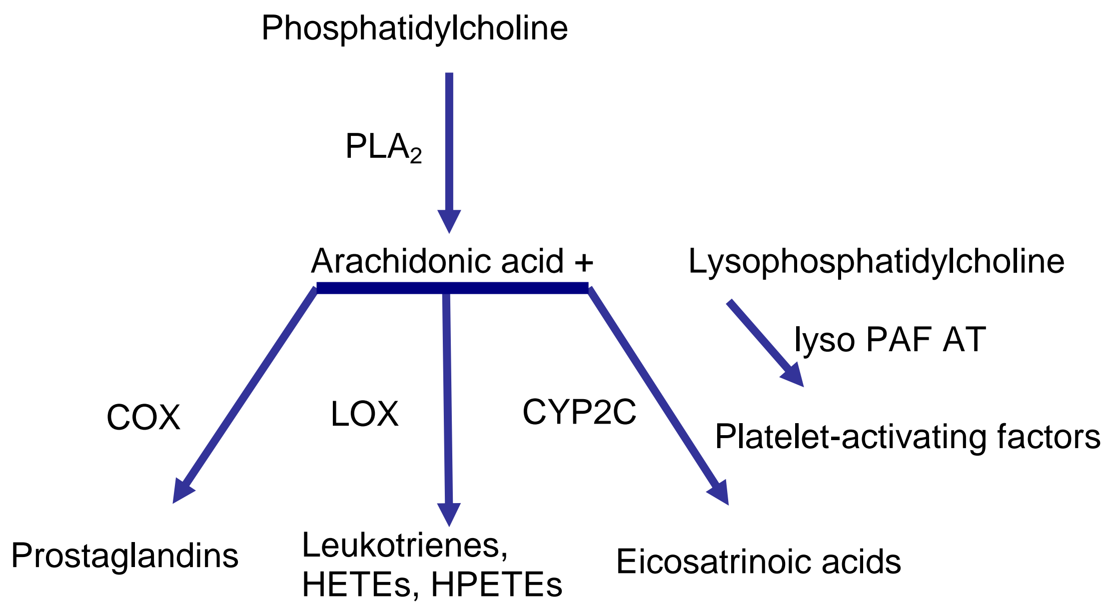


Figure 46 - Interaction and interconvertibility of lipid signaling pathways. Phospholipase A_2 (PLA_2), cyclooxygenase (COX), lipoxygenase (LOX), cytochrome P-450 2C (CYP2C), hydroxyeicosatetraenoic acid (HETE), hydroxyperoxyeicosatetranoic acid (HPETE), eicosatrinoic acid (EETs); acetyl-CoA lyso platelet-activating factor actyltransferase (lyso PAF AT), platelet-activating factor (PAF) [33]

Figure 46 describes the interactions of the PLA_2 lipid signaling pathway. This pathway can be subdivided into the arachidonic acid and lysophosphatidylcholine metabolism [35].
The release of arachidonic acid from membrane phospholipids is believed to be a key step in the control of eicosanoid production and involvement of inflammatory signaling [10, 22, 24, 47]. Fatty acids such as arachidonic acid are converted into eicosanoids through the action of a variety of prostaglandin and leukotriene synthesis mediated by cyclooxygenase, cytochrome P450 and lipoxygenase (Figure 46) [44, 63].
Lysophosphatidylcholine is metabolized to the platelet-activating factor (PAF). PAF is a potent phospholipid mediator that is produced by a variety of tissues and cells [21, 39, 124]. High levels of platelet-activating factor acetylhydrolase II, which play a role in the lysophosphatidylcholine metabolism, are expressed in sebaceous glands and in SZ95 sebocytes [94].

Data of this work indicate that Botmo GF 11-101 and Botmo GF 11-117 increase lipid synthesis in human SZ95 sebocytes. Surprisingly, stimulation of lipid synthesis was independent from PLA_2 enzymatic activity in Botmo GF 11 subfractions [136].
So far, there may be different mechanisms which can cause lipid stimulation in sebocytes after Botmo GF 11-101 and Botmo GF 11-117 treatment. Both mentioned

pathways, acachidonic acid and lysophosphatidylcholine, may influence the fatty acid production. Fatty acids can be released from phospholipids, sphingomyelin, ceramide, lysophospholipid, phosphatidic acid, lysophosphatidic acid, diacrylglycerol, ceramide-1-phosphate, platelet-activating factor, and sphingosylphosphorylcholine by the action of PLA_2. Many studies confirm that fatty acids activate neutral lipid production in SZ95 sebocytes [4, 87, 173].
It is known that PLA_2 are largely involved in skin changes like lipogenesis and inflammation. Botmo GF 11-101 and Botmo GF 11-117 may be interesting tools for the investigation of sebaceous lipogenesis and may be of help in developing therapeutic concepts to treat age-related skin dryness.

6.1.2 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS ACTIVATION BY PHOSPHOLIPASE A_2 PRODUCTS

To identify how *Bothrops moojeni* PLA_2, Botmo GF 11-101 or Botmo GF 11-117 stimulated lipid production in SZ95 sebocytes, different experiments were performed. To confirm the hypothesis that *Bothrops moojeni* PLA_2 fractions activate the fatty acid production which can act as PPAR ligand, Botmo PLA_2 fractions were tested for PPARα, δ or γ_2 activation in PPAR transiently transfected SZ95 sebocytes. Additionally, cytokine expression and release were tested in order to obtain more information about the inflammatory response after SZ95 sebocyte treatment with Botmo GF 11-101 or Botmo GF 11-117.
Botmo GF 11-101 and GF 11-117 led to different effects. Interestingly, with enzymatically inactive Botmo GF 11-101 no PPAR activation was detectable in SZ95 sebocytes. However, Botmo GF 11-117 was a significant PPARα activator (486%) in transiently transfected SZ95 sebocytes compared to control (100%) (Figure 33).
Furthermore, Botmo GF 11-101 and Botmo GF11-117 fractions did not express cytokines in significant amounts in SZ95 sebocytes.

Previous studies described that $cPLA_2$ is able to activate gene expression through PPARα and γ activation and binding to the PPAR response element in HepG2 cells [55, 113]. Other data indicated that $sPLA_2$ IIA released from rat mesangial cells by TNF-α stimulates its own expression via an autocrine loop involving $cPLA_2$ and PPARα [12]. However, there are no other data available so far suggesting a role of PLA_2 IIA in the process to activate PPAR. Additionally, the detailed mechanisms for their actions and the regulation of the key eicosanoid-forming enzymes in cells are not well understood.
Various $sPLA_2$, when added exogenously to rat mesangial cells, can dramatically up-regulate the expression of $sPLA_2$ IIA [12]. The role of exogenous $sPLA_2$ group IIA in regulating PLA_2 expression has been linked to $cPLA_2$ activation by $sPLA_2$. Activation of $cPLA_2$ by various $sPLA_2$ has also been reported in many other cells [9, 73, 80]. That involves $cPLA_2$ and intracellular lipid mediator formation to activate the nuclear receptor PPARα. In detail, these $cPLA_2$ activate arachidonic acid pathways which can further metabolize and produce eicosanoids. These resulting eicosanoids such as fatty acids, leukotrienes and prostaglandins are natural PPAR ligands [12, 20, 26, 33, 75, 182]. An additional study confirmed that $sPLA_2$ IIA has no influence to activate cytokine transcription [113].

However, various PLA_2 forms may play different roles in cell lipid metabolism and in the regulation of cell functions in various cells types. As mentioned before several cell culture models revealed that more than one PLA_2 is involved in cellular regulation and lipid messenger formation.

In this work both emphasized Botmo GF 11-101 and Botmo GF 11-117 activate lipid production in SZ95 sebocytes by obviously different biological pathways. Interestingly, the enzymatically inactive Botmo GF 11-101 was not able to activate PPAR. Enzymatically inactive Botmo GF 11-101 may stimulate lipid synthesis on a not yet investigated mechanism. However, enzymatically active Botmo GF 11-117 and *Naja mossambica mossambica* PLA_2 activate PPARα.

6.1.3 COMPARISON BETWEEN BOTMO GEL FILTRATION FRACTION, *HONEY BEE* AND *NAJA MOSSAMBICA MOSSAMBICA* PHOSPHOLIPASE A_2

Since first unexpected results identified PLA_2 in *Bothrops moojeni* venom gel filtration fractions showing lipid synthesis stimulation in SZ95 sebocytes, two different $sPLA_2$ (comparable snake *Naja mossambica mossambica* and *honey bee*) were tested for PPAR activity in transiently transfected SZ95 sebocytes. The aim was to investigate if the functions found with Botmo GF could also be achieved with other $sPLA_2$.
Naja mossambica mossambica $sPLA_2$ increased neutral lipid synthesis and polar lipid synthesis (Figure 22). *Naja mossambica mossambica* $sPLA_2$ showed a high significance of PPARα (306%) and a low significance of PPARδ (166%), PPARγ (127%) induction in transiently transfected SZ95 sebocytes (Figure 33). SZ95 sebocytes treated with *Naja mossambica mossambica* $sPLA_2$ released 10% more IL-8 compared to control cells which was not significant (Figure 26). Although *honey bee* PLA_2 stimulated lipid production in SZ95 sebocytes, no PPAR activation was observed.
Secreted PLA_2 can be divided in five different classes based on sequence criteria. Four classes share the catalytic activity but the fifth class of $sPLA_2$ group contains $sPLA_2$-like proteins [93]. These proteins bear 80% of structural homology towards class I/II enzymes but they are catalytically inactive due to one or more amino acid substitutions [142].
Class I and II enzymes are abundant in snake venom, human exocrine pancreas and human spermatozoa. *Honey bee* $sPLA_2$ belongs to class III. Compared to its class I/II relatives [74, 115], bee venom $sPLA_2$ is relatively insensitive [142]. This may explain the low lipid accumulation and no affinity to PPAR binding in SZ95 sebocytes by bee venom $sPLA_2$.
As mentioned before Botmo GF 11-101 is a non enzymatically active PLA_2 which may belong to $sPLA_2$ group V. Interestingly, a $sPLA_2$ from *Naja nigricollis* venom inhibited the prothombinase complex by non enzymatic mechanisms. Additionally, those special snake venom proteins without detectable enzymatic activity inhibited blood coagulation [74]. These observations of another PLA_2 isolated from a snake may prove the activity on lipid synthesis in sebocytes by a non enzymatic mechanism of Botmo GF 11-101 action.
Botmo GF 11-117 and *Naja mossambica mossambica* $sPLA_2$ lead to comparable results. The SZ95 experiments suggested that enzymatically active PLA_2 Botmo GF 11-117 is able to activate lipid synthesis in these cells by utilizing or activating the

arachidonic acid metabolism. Enzymatically active $sPLA_2$ IIA like Botmo GF 11-117 and *Naja mossambica mossambica* $sPLA_2$ showed a significant PPARα activation in SZ95 sebocytes. These enzymatically active PLA_2 may activate directly the arachidonic acid pathway and mediators which activate PPAR. The lipid synthesis and PPAR activation of PLA_2 in SZ95 sebocytes seem to be $sPLA_2$ class dependent.

6.1.4 INHIBITION OF PHOSPHOLIPASE A_2 IN SZ95 SEBOCYTES

PLA_2 activated significantly neutral lipid synthesis in SZ95 sebocytes. Recently it was hypothesized that the role of $sPLA_2$ group IIA in regulating PLA_2 expression has been linked to $cPLA_2$ activation by secreted PLA_2. To get more knowledge about the mechanism of lipid stimulation, which was suggested to involve the arachidonic acid pathway including $cPLA_2$, we tried to block the activation by a $cPLA_2$ inhibitor ($AACOCF_3$).
The inhibitory function of $AACOCF_3$ on PLA_2 activity resulted in suppression of the arachidonic acid release by $cPLA_2$ and the unspecific reduction of 5-LOX [36-38]. Furthermore, earlier studies showed that specific $cPLA_2$ inhibitors suppressed eicosanoids and PAF biosynthesis [16, 43, 170].

The $cPLA_2$ inhibitor $AACOCF_3$ was tested on SZ95 sebocytes to control lipid synthesis and reduce cytokine expression. $AACOCF_3$ treatment had no significant influence on the lipid content of SZ95 sebocytes. In addition, $AACOCF_3$ was not able to significantly suppress linoleic acid pre-stimulated lipids in SZ95 sebocytes (Figure 22). In *Naja mossambica mossambica* PLA_2 pre-stimulated SZ95 sebocytes AACOCF3 reduced neutral lipid synthesis by about 5%.
However, this study demonstrated a significant reduction of IL-6 (12%) and IL-8 (31%) expression by PLA_2 inhibitor $AACOCF_3$ in linoleic acid pre-stimulated SZ95 sebocytes (Figure 28).
Additionally, in another study $AACOCF_3$ reduced oleic acid induced IL-8 production [144]. $AACOCF_3$ inhibits production of lipid mediators, which stimulate the cytokines IL-6 and IL-8 [80]. These studies confirm our observations, which demonstrated significant IL-6 and IL-8 reduction.

In conclusion our results showed SZ95 sebocytes treated with $AACOCF_3$ did not block lipid synthesis but reduced cytokine expression, which is supported by several previous studies [144]. This experiment shows that $AACOCF_3$ is a very potent cytokine IL-6 and IL-8 suppressor in human SZ95 sebocytes.

6.1.5 5-LIPOXYGENASE INHIBITOR TREATMENT OF SZ95 SEBOCYTES LEADS TO A STRONGER REDUCTION OF CYTOKINE RELEASE THAN A GENERAL LIPOXYGENASE INHIBITOR

As a comparison to the general lipoxygenase (LOX) inhibitor (NDGA), the 5-lipoxygenase (5-LOX) inhibitor MK886 was tested in the context of lipid production and cytokine expression. NDGA treatments led to low, statistically not significant activation of lipid synthesis. MK886 slightly stimulated neutral lipid synthesis.

In general, LOX inhibitor (NDGA) treated sebocyte cytokine expression was not relevant compared to positive control (Biolip P51) treated cells. In comparison to NDGA, MK886 significantly reduced the release of the cytokines IL-6 (12%) and IL-8 (13%) expression in SZ95 sebocytes (Figure 28).

It has been reported that significant amounts of 5-LOX and low levels of 15-lipoxygenase (15-LOX) are expressed in SZ95 sebocytes both at the mRNA and protein level [4, 188]. The binding of leukotriene B_4 (LTB_4) to LTB_2-receptors activates 5-lipoxygenase in sebaceous gland cells, whereas 15-lipoxygenase-2 will not be activated by 15-hydroxyeicosatetraenoic acid (15-HETE) [148].
These activities include effects of LOX products which act directly as PPAR ligands, but may also act indirectly by modulation of MAP kinase phosphorylation of PPAR [79]. The LOX products, including LTB_4 [69], activate PPARα [179].

Generally, LOX inhibitors reduce LOX catalyzed reactions. MK886 reduces leukotriene syntheses LBT_4 [95, 195]. In an *in vivo* study, 5-LOX inhibition reduced inflammatory effects, which correlated with reduction of the sebaceous gland lipid content and the reduction of free fatty acids. However, leukotriene B_4 amounts in skin surface lipids were unchanged [184].
The observations of cytokine expression made with NDGA and MK886 treated SZ95 sebocytes correspond to results from *in vivo* studies which consider 5-LOX inhibition on human skin. In our *in vitro* experiments MK886 stimulated neutral lipid synthesis. This result is contrary to *in vivo* observations.
Both inhibitors did not block neutral and polar lipid synthesis in sebaceous gland cells. However, MK886 treatment of SZ95 sebocytes led to a stronger reduction of cytokine release than NDGA. MK886 significantly reduced IL-6 and IL-8 effects in SZ95 sebocytes. The 5-LOX inhibitor suppression effect of cytokines is supposed to be mediated by PPARα. These results suggest that MK886 may be more effective on SZ95 sebocytes compared to NDGA.

6.1.6 LIPOXYGENASE INHIBITOR ACTIVATES TRANSIENTLY TRANSFECTED PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ISOFORMS

PLA_2 activates the arachidonic acid metabolism which results in production of LOX and COX enzymes. These enzymes and fatty acid and their metabolites activate PPAR isotypes. Nordihydroguaiaretic acid (NDGA) was used as a non specific inhibitor for lipoxygenases. NDGA was reported to inhibit the hormonally induced differentiation in primary rat adipocytes [107, 150] and it was demonstrated that the glucose uptake stimulated by arachidonic acid in cells was blocked by NDGA [82, 107],
Surprisingly, NDGA as well as the combination of NDGA with arachidonic acid led to a significant activation of endogenous PPAR in empty expression vector transfected SZ95 sebocytes (Figure 34). Additionally, NDGA activated all PPARα, δ, γ_2 significantly in transiently transfected SZ95 sebocytes. A combination treatment with NDGA and arachidonic acid showed a synergetic PPAR activation compared to NDGA treatment alone. PPARα showed the strongest activation. This ligand binding study suggested that NDGA binds or interacts directly to PPARα, δ, γ_2 in transiently transfected SZ95 sebocytes (Figure 35).

Data from Thuillier et al. confirm that the general LOX inhibitor NDGA directly binds to PPAR [163]. When NDGA was tested on primary keratinocytes, NDGA blocked PPAR activity and differentiation of the cells. The reported reduction of PPAR activity induced by NDGA [163] contradicts the SZ95 sebocyte results. During SZ95 sebocyte experiments NDGA unexpectedly stimulated PPARα, δ, γ_2 activities. A notable observation by Yu et al. was that the general LOX reducing agent NDGA actually promotes LOX reactivity. The LOX products such as hydroxyeicosatetraenoic acid (HETE) and 15(S)-hydroperoxyeicosatetraenoic acid (HPETE) [177] may unspecifically trigger PPAR [177, 178] which may confirm the PPAR activation in our SZ95 sebocyte experiments.

The remaining PPAR activity thus might be triggered by other arachidonic acid metabolites such as LTB_4 and 15-HETE. In conclusion lipoxygenase products which rise after NDGA treatment may stimulate unspecifically PPARα, δ, γ_2.

6.1.7 INHIBITION OF CYCLOOXYGENASE IN ARACHIDONIC ACID ACTIVATED PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORα, δ, γ_2 TRANSIENTLY TRANSFECTED SZ95 SEBOCYTES

NS398 is a selective COX-2 inhibitor. PPARα, δ or γ_2 transiently transfected SZ95 sebocytes treated with arachidonic acid induced significant PPARα and PPARγ_2 activation. The treatment with NS398 alone did not activate PPAR in transiently transfected sebocytes, but NS398 inhibited and abolished arachidonic acid activated PPARα, δ and γ_2. The strongest PPAR inhibition induced by NS398 was observed in PPARα transiently transfected SZ95 sebocytes (Figure 35).

Data presented by Iwana et al. affirmed an increased formation of intracellular lipid synthesis after treatment with the COX-2 inhibitor NS398. This inhibitor reduces $PGF_{2\alpha}$ and PGE_2 levels but increases Δ15-PGJ_2 production and triacylglycerol synthesis which is connected to the augmentation of lipid droplet formation in sebocytes [56, 63, 188].
Additionally, NS398 was suggested to be associated with an increase in diacylglycerol acyltransferase activity in hamster sebocytes [63]. Diacylglycerol acyltransferase is involved in the lipogenesis pathway [149]. The involvement of prostaglandins in the formation of intracellular lipid droplets and the COX-2 depending pathway in hamster sebaceous glands were investigated.
In addition arachidonic acid induced accumulation of lipids in SZ95 sebocytes which activate PPAR [63, 173]. We observed that NS398 partially reduced a part of the arachidonic acid activated PPARα, δ and γ_2 in SZ95 sebocytes, which may result from decreased $PGF_{2\alpha}$ and PGE_2 levels [63]. Two studies confirmed the inhibition of PPARδ [176] and PPARγ by NS398 [123].

In summary, the NS398 does not act on PPAR in SZ95 sebocytes. NS398 treatment of arachidonic acid activated PPAR in PPARα, δ, γ_2 transiently transfected SZ95 sebocytes showed a suppression of PPAR activation, while still showing more activation than control cells. Since NS398 is a known $PGF_{2\alpha}$ and PGE_2 inhibitor, it

can be assumed that this effect is caused by $PGF_{2\alpha}$ and PGE_2 reduction. The inhibition of the PPAR ligand $PGF_{2\alpha}$ and PGE_2 may reduce PPARα, δ, γ_2 activation as demonstrated by the results presented in this work.
PPARα inhibition by NS398 in this study was more powerful than that of PPARδ and $\gamma_{2.}$ This provides evidence that the inhibition of the lipogenesis pathway, which exhibits proinflammatory activities and activation of PPARα [4, 63, 188] is highly interesting for further research manipulating the prostaglandin pathway to regulate PPAR activation.

6.1.8 ACTIVATION OF TRANSIENTLY TRANSFECTED PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORSα, δ, γ_2

Empty vector transfections were control experiments. No activation of empty vector transiently transfected sebocytes were expected. Interestingly, significant endogenous PPARδ and γ_2 activation was observed in empty vector transiently transfected SZ95 sebocytes.
As observed in previous studies endogenous PPARα, δ, and γ genes were expressed in sebocytes and sebaceous glands [19, 30, 132, 167]. One study described PPARβ as the predominant isoform in freshly isolated and cultured preputial rat sebocytes. PPARα and γ were also activated but to lower levels [72]. PPARβ/δ seems to be more important in the terminal differentiation stage of sebocytes [19, 72], Additionally, Alestas et al. observed PPARα and γ_1 are the predominantly activated PPAR isotypes in SZ95 sebocytes, while the expression of PPARδ and γ_2 was very low [4].

Furthermore, linoleic acid led to an unspecific activation of all transiently transfected PPAR subtypes whereas arachidonic acid only activated PPARα and γ_2. These data suggest that fatty acids like arachidonic acid and linoleic acid are unspecific PPAR ligands. Although the PPAR subtypes overlap in ligand recognition, there was a clear preference of the PPARα subtype for arachidonic and linoleic acid treated SZ95 sebocytes observed. Both fatty acids activated PPARα to the same extent as the specific PPARα ligand WY14643. Additionally, the activation of PPAR by linoleic or arachidonic acid was similar in both transiently transfected COS/7 cells and SZ95 sebocytes (Figure 30) (Figure 33). Linoleic acid and arachidonic acid significantly stimulated neutral lipid production in SZ95 sebocytes.
Numerous studies described linoleic acid as a non specific natural PPARα and β/δ ligand [129, 191]. Arachidonic acid is reported to be a natural PPARα ligand [4, 191]. Arachidonic acid derivatives like leukotriene and prostaglandin are the PPAR agonists of PPARα, β/δ, and γ isoforms [61, 71, 77]. However, data from Kersten, Wahli and Krey et al. confirm that these fatty acids are potent activators of all three PPAR and they observed a clear preference for the PPARα subtype activation [71, 77]. Fatty acids have been shown to regulate gene expression through direct interactions with PPAR [27, 77, 129].

This study confirmed that arachidonic and linoleic acid, which activate PPARα and γ_2, stimulated lipid production in SZ95 sebocytes [137]. Studies of Rosenfield corroborated that PPARα and γ activators were found to stimulate lipid droplet accumulation in cultured immature sebocytes [130-132]. PPARα mediates early

lipogenic steps to sebocyte function, and PPARγ may play a role in stimulating sebocyte lipogenesis. PPARβ/δ may contribute to lipid biosynthesis in sebocytes under certain circumstances, like in response to the high concentration found in sebum, which was synthesized by sebocytes [129].

In short, these data suggest that fatty acids unspecifically activate PPAR in a dose-dependent manner. Structurally diverse fatty acids may have the capacity to act directly on gene expression by binding to and activating PPARα, β/δ and γ_2 [77]. The fact that linoleic and arachidonic acid act as unspecific ligands of PPAR nuclear receptors in SZ95 sebocytes reveals that both may activate the PPAR pathway via the same mechanism. Possibly these fatty acids activate eicosanoid metabolism and the resulting products such as LTB_4, prostaglandin and 15-HETE activate PPAR.

6.1.9 CONCLUDING REMARKS

Data presented in this work revealed that *Bothrops moojeni* venom fractions GF 11-101 and GF 11-117 contain $sPLA_2$, which were identified for lipid regulating or stimulating activity in SZ95 sebocytes. Surprisingly, the newly identified fractions included both enzymatically active and inactive PLA_2 proteins.
The mechanism how $sPLA_2$ Botmo GF 11-101 or Botmo GF 11-117 activate and regulate lipid synthesis in SZ95 sebocytes is not completely understood. Interestingly, the enzymatically inactive Botmo GF 11-101 was not able to activate PPAR. Botmo GF 11-101 may stimulate lipid synthesis on a not yet investigated mechanism.
The PPAR activation of enzymatically active Botmo GF 11-117 suggests that Botmo GF 11-117 activates PLA_2, which induces eicosanoid and PAF production, followed by lipid stimulation and PPAR activation.
An important remaining question concerns the identification of the early cellular events that are used by the different $sPLA_2$. It is speculated that $sPLA_2$ activates $cPLA_2$. This $cPLA_2$ may move to the interior of the cell and activates the arachidonic acid or lysophosphatidylcholine metabolism. The resulting molecules of the metabolisms are COX, LOX, PAF and metabolites which stimulate lipid synthesis and PPAR [12]. Interestingly, $cPLA_2$ inhibitor $AACOCF_3$ was not able to block significantly PLA_2 or linoleic acid induced lipid stimulation in SZ95 sebocytes.
However, the exact pathway is still not identified. Botmo GF 11-101 and 11-117 might be interesting tools for the investigation of sebocyte lipogenesis and may be helpful to the development of therapeutic concepts for the treatment of age-related skin dryness.

7 OUTLOOK

This sebocyte screening model has been proven to be an excellent alternative to cell culture models using animal cells. Moreover, the composition of intracellular lipids from hamster sebaceous gland-derived cells is similar to that of human sebocytes with the exception that hamster sebocytes do not contain the human sebocyte-specific lipids, squalene and wax esters [62]. Cultured SZ95 sebocytes maintained properly preserve the major characteristic properties of human sebocytes *in vivo* and, therefore, the human sebocyte culture model is closer to the human in vivo situation [46, 185, 197].
The number of tests which have been carried out and the number of results produced show that this SZ95 sebocyte cell culture model is reliable, fast and usable to quickly run series of substance screenings [197].
The data presented in this work indicate that Botmo GF fractions 11-101 and 11-117 can increase lipid synthesis in human SZ95 sebocytes. Surprisingly, stimulation of lipid synthesis was independent of PLA_2 enzymatic activity in Botmo GF 11 subfractions.

To gain better insight into age-related lipid behavior of Botmo GF 11-117 and Botmo GF 11-101 treated sebocytes, the compounds should be tested on another SZ95 sebocyte model. A newly developed sebocyte culture model by Makrantonaki et al. [83, 85] may deliver information about lipid regulation in hormonally aged SZ95 sebocytes. During life IGF-1, GH, 17β-estradiol, progesterone, DHEA and testosterone levels in women and men decline. *In vitro* sebocytes treated with hormones that can be found in a 60-year old woman produce a lower level of lipids than those treated with a hormone cocktail of a 20-year old woman [83, 85]. It would be interesting to test Botmo GF 11-101 and 11-117 on these hormonally aged SZ95 sebocytes to determine whether Botmo GF 11-117 or Botmo GF 11-101 can activate lipid synthesis in “young” respectively “aged” sebocytes without cytotoxicity or apoptotic effects in equal measure.
Botmo GF 11-101 and Botmo GF 11-117 might be interesting subjects for the investigation of sebocyte lipogenesis and may be helpful to the development of therapeutic concepts for the treatment of age-related skin dryness.
Additionally, analysis of secreted or accumulated lipids from SZ95 sebocytes could provide insight into lipid composition and associated effects. It is reported that photo-oxidized lipids of sebocytes are inflammatory mediators. Sato et al. described a method to analyze the intracellular lipid content of sebocytes. Lipids were collected and then subjected to an automatic thin-layer chromatography Iatroscan [121, 134, 146].

In conclusion, sebocyte culture models provide new chances for further research on sebaceous gland disorders, dry skin and aged skin effects. They are useful tools for understanding the pathophysiological mechanisms of these skin effects. They also facilitate the search for biologically active ingredients, new pharmaceuticals and cosmetics for anti-ageing, seborrheic and dry skin treatment.

8 APPENDIX

8.1.1 LIPID INHIBITION BY GLYCYL-HISTIDYL-LYSINE

Glycyl-histidyl-lysine (GHK) is a liver like growth factor derived from parathyroid hormone. It is a very rare sequence in human proteins, mainly existing in inflammation-associated proteins and proteins of the extracellular matrix. This tripeptide has a high affinity to Cu^{2+} [29, 120]. In human plasma and wound areas, GHK is likely to exist as a mixture of GHK and GHK Cu^{2+}. GHK and GHK Cu^{2+} were investigated for wound healing effects and anti-inflammatory actions. These peptides heal broken and damaged skin and improve the quality of intact, undamaged skin [119].
Due to known actions of GHK on skin and hair follicles this copper peptide is used in cosmetic skin, hair care and dermatological skin renewing products. Formulations containing GHK Cu^{2+} increase skin elasticity, reduce wrinkles, and result in the removal of skin imperfections, such as sun damage marks, while producing an increase in subcutaneous fat cells [118].

Hydrocortisone is identified for wound healing effects, as well as suppression of neutral lipid content and cytokine release in SZ95 sebocytes [145]. For this reason, it was hypothesized that the wound healing properties of GHK may reduce neutral lipid synthesis and may suppress cytokine release in SZ95 sebocytes.
The results in this work document for the first time a lipolytic effect which was initiated by GHK. All tested GHK derivatives with and without Cu^{2+} showed a suppression of the lipid content in confluently grown SZ95 sebocytes. Only GHK demonstrated a significant activity in pre-stimulated SZ95 sebocytes, but not in non-stimulated SZ95 sebocytes. Treatment with GHK significantly inhibited the content of neutral lipids by 15%, but not of polar lipids which are representing cell membrane lipids in linoleic and arachidonic acid pre-stimulated, lipid accumulating SZ95 sebocytes.
Cell viability and proliferation remained unchanged for all data sets. Additionally, in this study GHK showed an inhibition of cytokine release, IL-6 by 13% and IL-8 by 15% [138].

Our results correspond to *in vivo* observations which have documented that GHK derivatives can show healing effects. The inflammatory response simulated with the pre-treatment of linoleic acid or arachidonic acid resulted in lipid increase as well as cytokine expression in SZ95 sebocytes. GHK treatment inhibited both inflammatory cytokines. Another study showed that the morphology of experimental wounds treated with GHK and GHK Cu^{2+} regenerated wound edge. The wound area was filled with hair follicles and sebaceous glands [109, 119]. The observed cytokine suppression was monitored by GHK Cu^{2+} treatment which has an inhibitory effect on IL-1β in rat pancreatic islets [171]. Also in cell culture experiment GHK Cu^{2+} reduces the secretion of TGF-α_1 in normal fibroblasts [92].
Until know, the mechanism of action of GHK and its derivatives is not identified. GHK insert their hydrophobic moieties on the external bilayers of liposomes leaving the tripeptide on the surface of vesicles. This behavior has no influence on the motion of phospholipid polar heads but reduces the penetration of other molecules, this way increasing the stability of the vesicles. The binding of GHK to phospholipid bilayers

was determined. Moreover, the influence of these peptidolipids on several physicochemical properties of liposomes was studied. Binding experiments indicate a high affinity of these peptidolipids for lipids ordered in liposomes [6].

In conclusion, the mechanism how GHK and GHK derivatives regulate lipid synthesis or cytokine expression is not fully identified. GHK negatively affects lipid synthesis when added to SZ95 sebocytes and reduces inflammatory cytokines in numerous experiments. These biological activities of GHK might be interesting for treatments to normalize skin lipid synthesis and against inflammations.

8.1.2 REDUCTION OF NEUTRAL LIPID PRODUCTION BY *VITEX AGNUS CASTUS* EXTRACT

Vitex agnus castus extracts showed a significant suppressing effect on neutral lipid production in SZ95 sebocytes. This experiment worked only reliably with freshly diluted *Vitex agnus castus* extract (100% water extract). This extract was not stable and lost its activity after about 3 days at 4°C.

In the traditional herbal pharmacology *Vitex agnus castus* extracts were used for the treatment of premenstrual syndromes. That suggests that compounds in the *Vitex agnus castus* extracts may be hormonally active.
A cell culture study revealed an estrogen action of *Vitex agnus castus* extract on steroid release. Jarry et al. identified the flavonoid apigenin, a selective phytoestrogen, which was recognized to act on ERβ [86]. Another study described the expression of ERα and ERβ in human SZ95 sebocytes [86]. This suggests that phytoestrogen may reduce neutral lipid synthesis in sebocytes. However, both mentioned studies worked with 68% ethanol extract which means that they isolated more phytoestrogen from *Vitex agnus castus* than we can with a water extract.

Up to know it has not been investigated how *Vitex agnus castus* water extract suppressed the neutral lipid synthesis in sebocytes. Neither the mechanism nor the exact compounds which act on the lipid production have been identified. *Vitex agnus castus* extracts may be interesting for further investigations to suppress lipid synthesis in sebaceous glands or SZ95 sebocytes.

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04/2004 – 06/2004 Research cooperation with Prof. Dr. Zouboulis, Dermato-endocrinology, Charité Berlin, Germany

- Sebocyte cell culture assays

Since 12/2003 Doctoral thesis at Pentapharm, University Basel, Switzerland

DIPLOMA DEGREE

03/2002 Diploma (Masters) in Chemistry at University Bremen, employed by Beiersdorf AG, Hamburg, Germany

04/2001 – 03/2002 Title of Diploma thesis: "Identification of porphyrins and other chromophore substances in human sebaceous secretions", Department for Cell Biology, Biochemistry and Biotechnology, University Bremen, Germany

- Spectroscopic UV Research into skin, Analysis of chromophore substances in human sebaceous secretion

- Skin Analysing Fluorescence Imaging Recorder; fluorescent view into the human follicle
- Microbiological identification of skin bacteria
- Analysis with HPLC and LC MSD
- Fluorescence Microscopy and Confocal Laser Scanning Microscopy
- Clinical studies with healthy volunteers

Higher Education

08/1997 – 06/1999	Internship: "DNA-Streptavidin conjugates as an agent for an immune sandwich assay", Centre of Environment, Science and Technology, University Bremen, Germany • Processing Protein Analysis using PCR and ELISA • Experience in the assembly of immune sandwich assays to prove antibodies
06/1995 – 09/1995	Protein analysis using PCR, Department of Chemistry, University Bremen, Germany
10/1991 – 03/2002	Studies of Chemistry and Biology, University Bremen, Germany
07/1991	German Abitur (A-level Germany)

Advanced Training

11/2006	Platform Skills – Professional Presentation, University Basel, Switzerland
10/2006	15th Congress of the European Academy of Dermatology and Venereology EADV, Rhodes, Greece
Since 04/2006	Mentoring program "*Woman into Industry*", Novartis, University Basel, Switzerland
11/2005	Project management for pharma industry, ETH Zurich, Switzerland
10/2005	14th Congress of the European Academy of Dermatology and Venereology EADV, London, Great Britain
06/2005	Pharma Business, -Marketing and Communication, ETH Zurich, Switzerland
03/2005	Quality and GMP, ETH Zurich, Switzerland
09/2004 – 10/2004	"Strategies and trends in pharmaceutical development and production", ETH Zurich, Switzerland

05/2003	14th International Congress for Bioengineering and the Skin, 8th Congress of the International Society for Skin Imaging, Hamburg, Germany
02/2003 – 03/2003	Project Management Training: Fuchs Management Training, Bremen, Germany
10/2002 – 12/2002	Intensive Language Training: Business-English, English Language Service, Bremen, Germany
04/2002 – 05/2002	Lecturing at Beiersdorf AG, Hamburg, Germany

RELEVANT WORK EXPERIENCE:

02/2000 – 06/2000	Administrator of the MADAM Database, Centre of Marine Tropic Ecology, University Bremen, Germany
10/1995 – 03/2001	Marketing: Promotion of products from the companies Sharp, Grundig, Fakir and Vilsa Mineral Water, Bremen, Germany

TEACHING EXPERIENCE:

02/1999 – 04/2000	Laboratory demonstrators for biology and chemistry students at the Department of Chemistry at University Bremen, Germany
02/1996 – 07/2002	Teacher for chemistry and biology students at an adult education centre, Bremen, Germany
10/1994 – 08/2001	Personal tutor for numerous students in the area of chemistry, mathematics and biology, Bremen, Germany

PUBLICATIONS:

Book chapter:

A.Herpens, SK.Schagen, S.Scheede: (2006) “Fluorescence Photography of Sebaceous Follicles“, Handbook of Non-Invasive Methods and the Skin, Taylor and Francis Group, London, New York, 2. Edition, Chapter 99, 853-860

Journals:

A.Herpens, SK.Schagen, S.Scheede, B.Timmermann, V.Schreiner, H.Wenck, K.-P.Wittern, R.Kruse, A.Klose: (2003), “SAFIR – Skin Analysing Fluorescence Imaging Recorder – An extended fluorescent view into the human follicle“ Skin Research and Technology, Denmark, Vol.9 (2), 184-185

CC.Zouboulis, SK.Schagen: (2006) “Akne – State of the Art” ; Georg Thieme Verlag, Stuttgart, Germany, Kompendium Dermatologie, 2; 12-13

A.Herpens, SK.Schagen, S.Scheede: (2006) "Evaluation of Comedogenic Activity by Skin Fluorescence Imaging Analysis (SAFIR)"; Bioengineering and the Skin

CC.Zouboulis; SK.Schagen, T.Alestas,: (2008) “The sebocyte culture - a model to study the pathophysiology of the sebaceous gland in sebostasis, seborrhoea and acne” , Archives of Dermatological Research, Vol.300 (3), 397-413

I.Kurokawa, W.Danby, Q.Ju, X.Wang, L.F. Xiang, L. Xia, W.Ch. Chen, I.Nagy, M.Picardo, D.H.Suh, R.Gancevicience, S.Schagen, F.Tsatsou, CC.Zouboulis: (2008) "Interesting developments in our knowledge of acne pathogenesis and treatment"; Experimental Dermatology, (in press)

SK.Schagen, AM.Perchuc, R.Voegeli, D.Imfeld, T.Schreier, CC.Zouboulis: (2009) “Phospholipase stimulate lipogenesis in SZ95 sebocytes” Sebaceous Gland, Acne, Rosacea and Related Disorders (in press)

Posters:

A.Herpens, S.Scheede, SK.Schagen, V.Schreiner, H.Wenck, K.-P.Wittern: (2003) “The Follicle Target – New Biophysical Tools for the Assessment of the Efficacy of Active Ingredients against the Signs of Impure Skin“, Journal of the European Academy of Dermatology and Venereology, Barcelona, Spain

A.Herpens, S.Scheede, SK.Schagen, V.Schreiner, H.Wenck, K.-P.Wittern: (2004) “The Follicle Target – New Biophysical Tools for the Assessment of the Efficacy of Active Ingredients against the Signs of Impure Skin“ Journal of the American Academy of Dermatology, San Francisco, USA

SK.Schagen, R.Voegeli, D.Imfeld, T.Schreier and CC.Zouboulis: (2007) “Lipid regulation in SZ95 sebocytes by liver-like growth factor”; Journal of the European Academy of Dermatology and Venereology, Vienna, Austria

SK.Schagen, AM.Perchuc, R.Voegeli, D.Imfeld, T.Schreier, CC.Zouboulis: (2007) “Phospholipase stimulate lipogenesis in SZ95 sebocytes”, LINK-AGE Meeting and IBMC Fall Symposium – The Free Radical Theory of Ageing – 50 Years beyond, 29 Nov - 1 Dec, Porto, Portugal

SK.Schagen, AM.Perchuc, R.Voegeli, D.Imfeld, T.Schreier, CC.Zouboulis: (2008) “Phospholipase stimulate lipogenesis in SZ95 sebocytes” ADF – XXXV Jahrestagung der Arbeitsgemeinschaft Dermatologische Forschung 28.Feb. – 1. Mar., Erlangen, Germany

SK.Schagen, R.Voegeli, D.Imfeld, T.Schreier, L.Michalik, W.Wahli, CC.Zouboulis: (2008) “COX-2 Inhibitor antagonizes PPAR activation in SZ95 sebocytes“; 1st European Congress for Dermato-Toxicology; 22nd - 25th Oct; Vaalsbroek, Netherlands

SK.Schagen; AM.Perchuc, R.Voegeli, D.Imfeld, T.Schreier, L.Michalik, W.Wahli, CC.Zouboulis: (2008) “Parts of the *Bothrops moojeni* snake venom activated lipid synthesis and PPAR α, β, γ_2 in transient transfected SZ95 sebocytes“; 1st European Congress for Dermato-Toxicology; 22nd - 25th Oct; Vaalsbroek, Netherlands

Poster Award Presentation:

SK.Schagen, AM.Perchuc, R.Voegeli, D.Imfeld, T.Schreier, CC.Zouboulis: (2008) ”Phospholipase stimulate lipogenesis in SZ95 sebocytes“, 2nd International Conference Sebaceous Gland, Acne, Rosacea and Related Disorders (September 13-16, 2008 - Rome)

Talks:

SK.Schagen, R.Voegeli, D.Imfeld, T.Schreier, L.Michalik, W.Wahli, CC.Zouboulis: (2007) Fatty acids are unspecific PPAR activators; 1st LINK-AGE / ESAAM Conference on Longevity, Ageing and Its Diseases, 21 - 23 June, Dessau, Germany

SK.Schagen, AM.Perchuc, R.Voegeli, D.Imfeld, T.Schreier, CC.Zouboulis: (2008) “Phospholipase stimulate lipogenesis in SZ95 sebocytes” ADE – XXXV Jahrestagung der Arbeitsgemeinschaft Dermatologische Endokinologie 27. Feb., Erlangen, Germany, Experimental Dermatology, 17, 624–632

SK.Schagen, AM.Perchuc, R.Voegeli, D.Imfeld, T.Schreier, L.Michalik, W.Wahli, CC.Zouboulis: (2008) “COX-2 inhibitor inhibited activated PPAR alpha, beta, $gamma_2$ in transient transfected SZ95 sebocytes.” EADV Journal of the European Academy of Dermatology and Venereology, Paris, France

SK.Schagen, A-M Perchuc , R. Voegeli, D. Imfeld, T. Schreier, L. Michalik, W. Wahli CC. Zouboulis (2009) “Parts of the *Bothrops moojeni* snake venom activated lipid synthesis and PPAR α, β, γ_2 in transient transfected SZ95 sebocytes”; ADE – XXXVI Jahrestagung der Arbeitsgemeinschaft Dermatologische Endokinologie 4 March, Heidelberg

SK.Schagen, A-M Perchuc , R. Voegeli, D. Imfeld, T. Schreier, L. Michalik, W. Wahli CC. Zouboulis (2009) “Parts of the *Bothrops moojeni* snake venom activated lipid synthesis and PPAR α, β, γ_2 in transient transfected SZ95 sebocytes”; 45. DDG-Tagung, Dresden