SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of novel neuroendocrine regulatory mechanisms in human skin

by Kinga Fanni Volascsekné Tóth

Supervisor: Dr. habil. Attila Oláh, MD, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2024

Investigation of novel neuroendocrine regulatory mechanisms in human skin

By Kinga Fanni Volascsekné Tóth
molecular biologist (MSc)

Supervisor: Dr. habil. Attila Oláh, MD, PhD

Doctoral School of Molecular Medicine, University of Debrecen

Head of the **Defense Committee**:	János Szöllősi, PhD, DSc, MHAS
Reviewers:	Zsuzsanna Bata-Csörgő, PhD, DSc
	Árpád Szöőr, PhD
Members of the Defense Committee:	Lajos Vince Kemény, PhD
	Zsolt Dajnoki, PhD

The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
at 1 pm, 7th of June, 2024.

Introduction and literature review

The skin is one of the largest organs of the human body and plays a crucial role in protecting our body. Forming a complex barrier system, it works closely with the immune system to protect against chemical, physical/mechanical, and biological threats from our environment. For many years, our group has been investigating the (patho)physiological processes of the skin and how the different cell types contribute to the development or improvement of inflammatory and/or pruritic diseases. Another core area of research in our laboratory is the investigation of the endocannabinoid system (ECS). The ECS is a complex physiological regulatory system whose receptors and endogenous ligands are found almost everywhere in the body, including the skin. In addition to the "classical" members of the ECS, members of the transient receptor potential (TRP) ion channel family, which also function as ionotropic cannabinoid receptors, are also present in the skin. Because we have recently shown that the thermosensitive TRPV1, -2, and -4 ion channels, which are mainly permeable to Ca^{2+}, are expressed in human sebaceous gland cells, we have investigated the expression and role of the fourth "warm-sensitive" TRPV channel, TRPV3.

In the second part of our experiments, we investigated the effects of fluoxetine (FX), an antidepressant not directly related to the cannabinoid system, which has been safely used in clinical practice for decades, on human epidermal keratinocytes, with a particular emphasis on the possible anti-inflammatory effects of the molecule and the possibility of its dermatological re-positioning.

Sebaceous glands and acne

The most common condition associated with abnormal sebaceous gland function is acne. Its pathogenesis is a complex process involving four main factors: seborrhea, i.e. increased production of sebum with an altered quality and quantity, abnormal keratinization of the duct, proliferation of pathogenic *Cutibacterium acnes* (formerly *Propionibacterium acnes*) strains in the

pilosebaceous unit and associated pathological inflammatory processes. In addition to the above, androgen hormones play an important role in the pathogenesis of acne, as increased sebum production is often associated with the overproduction of male sex hormones. Acne typically appears on the face but it is also common on the back and chest. Acne may present as a variety of non-inflammatory lesions, i.e., open or closed comedones (blackheads), and inflammatory lesions usually in the form of papules, pustules, or cysts. Successful treatment of acne often requires a combination of different treatments. The most effective currently available anti-acne agent is isotretinoin; however, it can have serious side effects (including teratogenicity). This explains why there is a strong worldwide demand for identifying new therapeutic options with a more favorable side effect profile that may be able to effectively alleviate the symptoms of different forms of acne.

The role of TRP channels in sebaceous glands

TRP channels are expressed in various cell types in the skin, including keratinocytes, sensory neurons, melanocytes, and various immune cells, and play a role in the regulation of many physiological processes. Their importance is demonstrated by a growing body of evidence indicating that dysfunction of these TRP channels can lead to diseases and conditions such as chronic pain, itching, various forms of dermatitis, vitiligo, alopecia, wound healing disorders, various skin tumors, and barrier damage.

In addition to the above, our group has been investigating the functional role of TRP channels in several processes of skin biology. We have recently shown that stimulation of TRPV3 through the NF-κB pathway induces a strong inflammatory response in human epidermal keratinocytes. Moreover, several lines of evidence (including our own data) suggest that overexpression and hyperactivity of TRPV3 in epidermal keratinocytes contribute significantly to the pathogenesis of various forms of dermatitis and to the excruciating itch that is

characteristic of atopic dermatitis (AD). Although the above data suggest that some TRPV channels play an important role in the regulation of skin and more specifically sebocyte biology, experimental data on TRPV3 in human sebaceous glands were not available at the time of our experiments. In light of this, in the first half of our experiments, we focused on investigating the expression and role of TRPV3, already extensively studied in the skin, in human sebocytes.

The integrity of the skin's barrier functions is essential for the body's survival. Its dysfunction or impairment can lead to extremely common diseases such as AD, also known as eczema. AD is a highly complex disease, which can be classified into several different clinical subtypes and symptomatic and pathogenic subgroups. It is well known that disturbances of the physicochemical, microbiological, and immunological barriers play an important role in the development of AD symptoms. Among others, impaired differentiation of epidermal keratinocytes and thus defective physicochemical barrier function, genetic predisposition (e.g., filaggrin mutations) or other factors, including abnormal lipid production in the skin, increased skin surface pH, abnormal changes in the skin microbiota, and abnormal T_h2/T_h22-dominated immune response are all present in AD. In addition to these processes, the expression of Toll-like receptor (TLR)-3 has also recently been found to be associated with AD symptom severity. TLR activation may not only lead to the release of antimicrobial peptides and various cytokines but may also trigger the production of other mediators important for skin (patho)physiological processes, such as itch mediators. In this respect, the aforementioned TLR3 expressed in keratinocytes is of particular importance. This is why we have chosen the polyinosinic-polycytidylic acid (p(I:C)) - TLR3 inflammatory model in the present experiments, as TLR3 is involved in the pathogenesis of AD and in the development of itch and the “itch-scratch cycle” that significantly aggravates

symptoms, and may also play a role in other itch-associated pathologies besides AD.

FX is a selective serotonin reuptake inhibitor (SSRI). It is an antidepressant that has been used in the clinical practice for decades. It is important to note that recent (mostly animal) data suggest that FX may exert significant anti-inflammatory effects in various organs (including the skin), may exert anti-pruritic effects, and can promote (re)pigmentation of micro-dissected human hair follicles. It is important to note that the anti-inflammatory effects of FX are in most cases likely to be independent of the classical serotoninergic signaling and may instead be mediated through interactions with other signaling pathways. Such possible alternative pathways include NLRP3 inflammasome activation, nitric oxide production, as well as inhibition of the phosphatidylinositol 3-kinase (PI3K) pathway.

FX has a more favorable safety and side effect profile in clinical use compared to other SSRI molecules. For this reason, and in light of the above-mentioned anti-inflammatory, anti-itch, and other “non-classical” effects, it is not surprising that the idea of drug repurposing has been raised in several cases. Considering all this, in the present study, we aimed to further explore the potential anti-inflammatory effects of FX in human epidermal keratinocytes.

Aims

In light of the literature detailed above and our previous findings, we sought to answer the following questions:

1. Is TRPV3 expressed on human sebocytes and, if so, what is its role in the regulation of typical biological processes (e.g., fatty lipid synthesis) and immune phenotype of these cells?

2. How does FX affect the inflammatory processes of human epidermal keratinocytes and what might be the mechanism of action?

Materials and Methods

Culturing of human immortalized SZ95 sebocytes

SZ95 sebocytes were cultured in Sebomed® Basal Medium. This basal medium was supplemented with heat-inactivated fetal bovine serum (FBS), human recombinant epidermal growth factor, $CaCl_2$, and MycoZap™ Plus-CL antibiotic. Cultured cells were maintained in a 37 °C incubator in a humidified environment with 5% CO_2. Possible *Mycoplasma* contamination of the cultures was regularly checked using the MycoAlert™ PLUS Mycoplasma Detection Kit and negative results were obtained in all cases. Cell culture was performed by *Dr. Magdolna Szántó, Anita Galicz, and Kinga Fanni Volascsekné Tóth*.

Culturing of primary normal human epidermal keratinocytes (NHEK)

NHEKs were isolated from dermatologically healthy individuals who had undergone surgical intervention. The experiments were approved by the Regional and Institutional Research Ethics Committee of the University of Debrecen and the Hajdú-Bihar County Government Office (IDs: IX-R-052/01396-2/2012, IF-1647-2/2016, IF-1647-9/2016, IF-778-5/2017, 61566-5/2021/ECIG, DE RKEB/IKEB 4988-2018), in compliance with the guidelines of the Declaration of Helsinki. Donors gave written informed consent for the use of their samples for research purposes. Cells were cultured in serum-free EpiLife culture medium supplemented with keratinocyte growth-promoting HKGS. NHEKs were cultured at 37 °C in a humidified atmosphere with 5% CO_2 and only keratinocytes below passage number 4 were used in the experiments. Cell isolation and culture were performed by *Judit Szabó-Papp, Anita Galicz, Dorottya Ádám, Mónika Barotáné Kovács, and Kinga Fanni Volascsekné Tóth*.

Culturing of the HaCaT keratinocyte cell line

Human immortalized HaCaT keratinocytes were cultured at 37 °C in a humidified environment with 5% CO_2 in DMEM medium supplemented with heat-inactivated FBS and antibiotic solution. Possible *Mycoplasma* contamination of the cultures was regularly checked using MycoAlert™ PLUS Mycoplasma Detection Kit and negative results were obtained in all cases. Culturing of cells was performed by *Judit Szabó-Papp, Anita Galicz, Mónika Barotáné Kovács, Dorottya Ádám, and Kinga Fanni Volascsekné Tóth*.

Immunofluorescent labeling

Cells were fixed in ice-cold acetone and permeabilized with 0.1% Triton-X-100 detergent dissolved in phosphate-buffered saline (PBS). After washing with PBS and blocking with 1% bovine serum albumin dissolved in PBS for 30 min, cells were incubated with TRPV3 specific primary antibodies in blocking solution at 4 ^{o}C overnight. As a fluorescent label, the cells were then incubated with fluorescein isothiocyanate-conjugated secondary antibodies, and 4',6-diamidino-2-phenylindole was used as nuclear staining. TRPV3 expression was examined using a Nikon Eclipse E600 fluorescence microscope. Cells labeled with the omission of the primary antibody were used as negative controls. Immunofluorescence labeling was performed by *Dr. Magdolna Szántó.*

Detection of TRPV3 expression in sebaceous glands (immunohistochemistry)

The expression of TRPV3 in sebaceous glands was detected in formalin-fixed paraffin-embedded samples from sebaceous gland-rich skin areas of patients diagnosed with trichilemmal (pilar) cysts at the Kenézy Gyula University Hospital (Debrecen). After blocking, tissue sections were incubated with a specific primary antibody against TRPV3. The expression of the channel was detected by using HRP-conjugated EnVision+ system according to the manufacturer's instructions, and the presence of TRPV3 was visualized using 3,3'-diaminobenzidine. The

nuclei were labeled with hematoxylin, and finally, the sections were covered with an appropriate mounting medium. Sections labeled with the omission of the primary antibody were used as negative controls, while the epidermis was used as a tissue positive control on the sections. TRPV3 expression was examined under transmitted light using the previously mentioned Nikon Eclipse E600 fluorescence microscope (Nikon). Immunohistochemical labeling was performed by *Dr. Ágnes Pór and Dr. Ilona Kovács.*

RNA isolation, reverse transcription, and quantitative, real-time polymerase chain reaction (Q-PCR)

Cells were harvested with 500 µl Trizolate or TRIzol reagent. A NanoDrop-1000 spectrophotometer was used to determine the RNA content of our samples. RNA was reverse transcribed to cDNA using Ambion™ reverse transcription kits. Q-PCR reactions were performed using TaqMan assays and Gene expression Master Mix. Gene expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin A (PPIA), and 18S ribosomal RNA (RNA45S) was used as internal controls. Q-PCR reactions were performed on 384 well PCR plates in a LightCycler® 480. Each reaction was performed with three technical replicates. The relative expression of each gene was expressed as mean±SD normalized to the gene expression values of the internal control using the ΔCT method. The measurements were performed by *Dr. Magdolna Szántó and Kinga Fanni Volascsekné Tóth*.

Western blot

Cells were harvested in lysis buffer that contained protease inhibitor cocktail and PhosSTOP reagent. Protein concentration was determined using a BCA protein assay kit. The samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. 10% Mini Protean TGX gels were loaded with equal amounts (15 µg or 30 µg) of protein per lane. Depending on the purpose of the experiment, the membranes were probed with primary antibodies raised

against human TRPV3, p-IκBα, or p-p38 MAPK overnight at 4°C. As secondary antibodies, a horseradish peroxidase (HRP)-conjugated goat antibodies were used. The membranes were incubated with the secondary antibodies at room temperature for 1 hour. The immunoreactive bands were visualized using chemiluminescent SuperSignal™ West Pico PLUS and Femto PLUS Chemiluminescent Substrate kit in a KODAK Gel Logic 1500 Imaging System. To assess equal loading, expression of β-tubulin was determined as described above. Semi-quantitative densitometric analysis of signals was performed using *ImageJ 1.52a* software. Western blot experiments were performed by *Dr. Magdolna Szántó, Dorottya Ádám, József Arany* and *Kinga Fanni Volascsekné Tóth*.

Quantification of the intracellular lipid content (Nile Red labeling)

SZ95 sebocytes were plated in 96-well plates with "black well, clear bottom", and incubated with Nile Red solution (1 μg/ml). After half an hour, the fluorescence intensity was measured at nine points per well in the well-scan mode at 485 nm excitation and 565 nm detection wavelengths, proportional to the amount of neutral (sebaceous) lipids, using a FlexStation 3 multimode plate reader. Nile Red labeling was performed by *Dr. Magdolna Szántó, Dr. Attila Oláh and Kinga Fanni Volascsekné Tóth.*

Investigation of viability (MTT-assay)

The supernatant of the cell was removed, and MTT solution (dissolved in PBS; final concentration: 0.5 mg/ml) was added to the cells. The cells were incubated with this solution at 37 °C for 3 hours. After removal of the solution, the crystals were dissolved in solubilizing solution, and the absorbance was measured at 565 nm using the FlexStation 3 as mentioned above. When the effects of FX were investigated, the evaluation was slightly different. In these experiments, absorbance was also measured in the empty wells of the plates, and the average of the values of these wells was taken as background and subtracted

from the absorbance values measured for all other wells. The background-corrected values were then normalized to the solvent-treated control and a curve was fitted to the resulting values using the "non-linear curve fitting" function of GraphPad Prism 9.3.1 (471) software. Cell viability was examined by *Dr. Magdolna Szántó, Dr. Attila Oláh and Kinga Fanni Volascsekné Tóth.*

Cell count analysis (CyQUANT assay)

Epidermal keratinocytes were plated in "black well, clear bottom" 96-well plates at 5,000 cells/well, and treatments were performed. The experiments were performed according to the manufacturer's protocol, and the fluorescence intensity was measured at nine points per well using a FlexStation 3 instrument with an excitation wavelength of 480 nm and a detection wavelength of 520 nm in "well scan" mode. The CyQUANT assay was performed by *József Arany, Dorottya Ádám and Kinga Fanni Volascsekné Tóth.*

Fluo-4 AM-based fluorescence Ca^{2+} measurement

Sebocytes were plated into 96-well plates with black walls and transparent bottoms, and the measurements were performed in Hank's solution. In cases where the effects of the treatments were tested in nominally Ca^{2+}-free Hank's solution, an equimolar amount of glucose was added to the Hank's solution instead of $CaCl_2$. The measurement was carried out using FlexStation 3 in "FLEX mode" at 37 °C. Fluorescence intensity was monitored continuously using excitation wavelengths of 490 nm and detection wavelengths of 520 nm. Fluo-4 AM-based fluorescence Ca^{2+} measurements were performed by *Dr. Magdolna Szántó, Dr. Attila Oláh and Kinga Fanni Volascsekné Tóth.*

Selective gene silencing (siRNA transfection)

siRNA-transfection was carried out in Opti-MEM® medium using Lipofectamine RNAiMax Reagent. As a control, a "Stealth RNAi Negative Control Med GC" double-stranded RNA construct ("scrambled RNA construct"

[SCR]) was used. Silencing efficiency was checked by western blot and Q-PCR. Selective gene silencing was performed by *Dr. Magdolna Szántó and Kinga Fanni Volascsekné Tóth*.

Cytokine array

Supernatants were analyzed using the "Proteome Profiler Human XL Cytokine Array Kit" according to the manufacturer's protocol. Semi-quantitative densitometric analysis of the signals was performed using *ImageJ 1.52a* software. For the evaluation of the results, the average of the negative controls on each membrane was taken as the background signal intensity and subtracted from the raw values. Then, the means of 2-2 technical replicates of each molecule were normalized to the data obtained for the control culture, and the ≥1.5-fold change due to p(I:C) treatment was considered as the relevant effect. The experiment was performed by *Kinga Fanni Volascsekné Tóth.*

Investigation of cytokine release (ELISA)

We measured the release of IL-1α, IL-1β, IL-6, IL-8 and TNF-α cytokines using OptEIA™ Set Human ELISA kits following the manufacturer's protocol. The experiments were performed by *Dr. Magdolna Szántó and Kinga Fanni Volascsekné Tóth.*

Investigation of endothelin release (pan-endothelin ELISA)

The endothelin content of supernatants was determined using the "Endothelin Pan Specific DuoSet ELISA" kit, following the manufacturer's protocol by *Dr. Attila Oláh, Dorottya Ádám and Kinga Fanni Volascsekné Tóth*.

Investigating the differentiation of sebocytes by flow cytometry

SZ95 sebocytes were loaded with Nile Red. Next, cells were analyzed using a NovoCyte Flow Cytometer with a 488 nm excitation laser. The fluorescence signal of Nile Red and the side scatter (correlating with granulation) were detected

simultaneously and the data were evaluated using *FlowJo* software. The experiment and the analysis of the results were performed by *Kinga Fanni Volascsekné Tóth, Zsófia Pénzes and* Dr. *Attila Gábor Szöllősi.*

MitoSOX Red labeling

Cells were loaded with 5 μM MitoSOX Red superoxide indicator dissolved in a serum-free, pre-warmed medium. Cells were then washed three times with the medium used for keratinocyte culture (DMEM) and treated as indicated. After incubation for 30 min at 37 °C, fluorescence was measured at 510 nm excitation and 580 nm emission wavelengths using a FlexStation 3 plate-reader in the "well scan" mode. After subtraction of the background signal (the signals measured on unloaded cells in the same plate), the average of these measurements was plotted as a data point in relative fluorescence units. The experiment and the evaluation of the results were performed by *Dr. Attila Oláh* and *Kinga Fanni Volascsekné Tóth*.

RNA-Seq method

HaCaT keratinocytes were collected following the appropriate 24-hr treatments. mRNA sequencing was performed on an Illumina sequencing platform. Only samples with RNA Integrity Number (RIN value) >7 were used in the library preparation process. The raw sequencing data (fastq) were aligned to the human reference genome version of GRCh38 using the HISAT2 algorithm and BAM files were generated. Further analysis was performed using StrandNGS software (www.strand-ngs.com). BAM files were imported into DESeq software and normalization was performed using the DESeq algorithm. A moderated T-test was used to identify genes differentially expressed in response to treatments. The pathways analysis was performed with g:Profiler (version e109_eg56_p17_1d3191d) using the "Ordered query" function and the default settings recommended in the website against the "GO:molecular function"

database. Raw data from the RNA-Seq analysis are available in the NCBI SRA database (http://www.ncbi.nlm.nih.gov/bioproject/1022043).

RNA samples were prepared by *Kinga Fanni Tóth*. Sequencing was performed by *Dr. Szilárd Póliska*. Data analysis was performed by *Dr Szilárd Póliska, Dr Attila Oláh* and *Kinga Fanni Volascsekné Tóth*.

BioMAP® Diversity PLUS® profile analysis

The BioMAP® Diversity PLUS® panel is used to explore the bioactivity profile of the substances of interest. The effects of multiple concentrations of a substance are tested in 12 model systems of primary cells representing different organ systems of the human body. The overall effects on standard experimental endpoints, predefined in each model system, gives the activity profile for different concentrations of the substance, which is compared with the activity profiles of substances in the company's database at the end of the experiment and the degree of similarity (using appropriate mathematical analysis) is determined. The BioMAP technology is protected by patents US10018621B2, US9734282B2, US8697387B2, US8467970B2, US8019551B2, US7912651B2, US7908089B2, US7266458B2, US6763307B2 and US6656695B2. Experiments and analyses were performed by *Dr. Jennifer I. Drake and Dr. Alison O'Mahony.*

Molecular docking (molecular modeling)

The crystal structure of the GDC0941-PI3K complex (PDB ID: 3DBS) was obtained from the Protein Data Bank database. The analysis was performed using PyMOL 1.7.5.0 with AutoDockTools (ADT) and MGLTools 1.5.7191. The 3D structure file of FX was obtained from the National Biotechnology Information Center PubChem Compound database and all simulations were performed on an ASUS GL502V workstation by *Dr. Yesid A. Ramirez.*

PI3K activity assay

PI3K activity was measured using the PI3K-Glo™ Class I Profiling Kit according to the manufacturer's instructions. Luminescence was determined using a FlexStation 3 multimode reader, and for some preliminary experiments, measurements were made using an Envision® 2105 Multimode Plate Reader. After correction by the background value, the results were expressed as a percentage of vehicle control considered to be 100%. Outliers were identified using the ROUT method. The experiments and analysis were performed by *József Arany, Dr. Attila Gábor Szöllősi, and Dr. Attila Oláh.*

Statistical analysis

Data were analyzed and plotted using IBM SPSS Statistics 23.0 and OriginPro 9 for the experiments on the sebocytes and using GraphPad Prism 9.3.1 in the FX-related experiments. When TRPV3 was tested in the sebocytes, two-tailed unpaired t-tests (pairwise comparison) and one-way ANOVAs were performed, followed by Dunnett's (comparison with a control group) or Bonferroni (multiple comparisons) *post hoc* tests. To investigate the biological effects of FX on keratinocytes, the distribution of data was tested using Anderson-Darling or Shapiro-Wilk tests, depending on the sample size. For normal distributions, two-tailed unpaired t-test (paired comparisons) or one-way ANOVA followed by a Šídák *post hoc* test (multiple comparisons) was used, whereas, for non-normal distributions, a two-tailed Mann-Whitney test (paired comparisons) or Kruskal-Wallis test followed by a Dunn *post hoc* test (multiple comparisons) were used. $P<0.05$ was considered a significant difference in all cases. The statistical analysis and the preparation of the figures were performed by *Kinga Fanni Volascsekné Tóth, Dr. Magdolna Szántó, Dr. István Balázs Tóth and Dr. Attila Oláh*.

Results

TRPV3 is expressed in human sebaceous glands

First, by immunohistochemical analysis, we have shown that TRPV3 is expressed not only in the epidermis but also in sebaceous glands. Moreover, our results showed that peripherally located undifferentiated cells exhibited higher immunopositivity than centrally located, more differentiated cells, suggesting that TRPV3 may play a role in the regulation of sebocyte maturation.

TRPV3 exhibits an expression pattern similar to that observed in sebaceous glands in human SZ95 sebocytes

Considering the aforementioned lack of an appropriate animal models for human sebaceous glands, and the considerable objective difficulties in studying primary sebocytes, we performed our experiments using the SZ95 sebocyte cell line, which is a widely used model for human sebocytes. We found that sebocytes express TRPV3 both at the protein and mRNA levels. Moreover, in line with the results obtained from immunohistochemical staining, the expression of the ion channel was found to be lower in post-confluent differentiated cultures than in rapidly proliferating pre-confluent cell cultures.

Activation of TRPV3 induces Ca^{2+} signals in SZ95 sebocytes

After describing the expression pattern, we performed functional measurements to elucidate the biological role of TRPV3. As mentioned above, in our previous studies we found that TRPV3 is expressed on human epidermal keratinocytes in a functionally active form, i.e., as a predominantly Ca^{2+}-permeable ion channel. Thus, in the present experiments, we also aimed to investigate the effects of a synthetic (2-aminoethoxydiphenylborane [2-APB]) and a plant-derived (carvacrol) activator of TRPV3 on human sebocytes. As in keratinocytes, TRPV3 activators induced a significant increase in intracellular Ca^{2+} concentrations in sebocytes. However, Ca^{2+} signals were almost completely

abolished in the presence of the general TRP channel inhibitor ruthenium red, whereas selective antagonists of the TRPV1 and TRPV4 ion channels (AMG9810 and HC067047, respectively), which are structurally similar to TRPV3 and are also functionally expressed on cells, did not affect the generated Ca^{2+} signals.

Unfortunately, at the time of our experiments, selective TRPV3 activators and antagonists were not commercially available, so we also investigated the TRPV3 specificity of the effects of 2-APB and carvacrol by RNA interference-based selective "gene silencing". As already mentioned in the "Materials and Methods" section, sebocytes were transfected with small interfering RNA (siRNA) targeting TRPV3 mRNA, which resulted in a significant decrease in the expression of the channel mRNA and protein level expression by post-transfection day 2 compared to the cells transfected with non-sense RNA ("scrambled"; SCR).

Although the method was not able to completely abolish TRPV3 expression, our functional measurements showed that the amplitude of the agonist-induced Ca^{2+} signals and the slope of the ascending limb of the signals were significantly reduced in the "gene silenced" cells. This implies that the effects of 2-APB and carvacrol on Ca^{2+} homeostasis in human sebocytes are most likely predominantly mediated by TRPV3.

TRPV3 agonists reduce sebocyte viability in a concentration-dependent manner

We next wanted to know how the pharmacological activation of TRPV3 affects the typical biological processes of sebocytes. First, we investigated the effect of TRPV3 activators on viability. We found that higher concentrations of the activators reduced the number of viable cells over the course of 24-hr treatments, while at lower concentrations (that still induced clear Ca^{2+} signals) they exerted no cytotoxic effects. Based on these results, the biological effects of

these effective but non-cytotoxic concentrations (2-APB: 150 μM; carvacrol: 500 μM) were further investigated in the following experiments.

TRPV3-mediated Ca^{2+} signaling inhibits sebaceous lipogenesis

As discussed previously, activation of TRPV1 and TRPV4 ion channels, which are highly similar to TRPV3, significantly reduces sebaceous lipogenesis induced by various lipogenic agents (e.g. arachidonic acid [AA]) in human sebocytes, and we were interested to see how TRPV3 affects lipid synthesis. To investigate this, we activated TRPV3 using the above defined effective, but non-cytotoxic concentrations of 2-APB and carvacrol. The treatments significantly reduced AA-induced sebaceous lipid synthesis in cells transfected with non-sense RNA (scr), whereas they had a significantly smaller effect in TRPV3-silenced sebocytes. We also observed a small but significant increase in the effect of AA following TRPV3 gene silencing, suggesting that basal, homeostatic activity of the channel also negatively regulates AA-induced lipogenesis.

It is important to note that the effect of TRPV3 agonists was not limited to AA-induced lipid synthesis. The activators similarly inhibited the lipogenic effects of the endocannabinoid AEA (CB_2 and ERK1/2 MAPK activation) and the linoleic acid-testosterone combination (PPAR activation), which act through different mechanisms than AA (PKCδ activation), and also slightly reduced basal sebaceous lipid production in non-transfected sebocytes. All this suggests that TRPV3 is a potent negative regulator of sebaceous lipid production and that its activators (like TRPV4 activators) induce universal lipostatic effects.

The TRPV3 agonist carvacrol reduces AA-induced lipid synthesis, and this effect can be prevented by ruthenium red

Consistent with the above results, we also found by flow cytometry that carvacrol (although it did not affect cell granularity in either control conditions or AA-treated cells) selectively inhibited AA-induced intracellular lipid

accumulation. This effect could be prevented by the general TRP channel antagonist ruthenium red, confirming that indeed a TRPV channel (most likely TRPV3) may mediate it.

The mRNA expression of PPARγ and NRIP1, two positive regulators of sebaceous lipid synthesis, is reduced by carvacrol

To assess the molecular background of this phenomenon, we further investigated the mRNA expression of the important positive regulators of sebaceous lipid production, namely peroxisome proliferator-activated receptor (PPAR)-γ and nuclear receptor interacting protein (NRIP)-1. We have demonstrated that carvacrol can down-regulate the expression of both molecules at the mRNA level, suggesting that down-regulation of PPARγ and NRIP1 may be involved in the lipostatic effect of TRPV3 activators.

Carvacrol enhances the expression and release of certain inflammatory cytokines in a partially TRPV3-dependent manner

Besides lipid production, sebaceous glands also contribute to the regulation of cutaneous immune processes. Importantly, activation of TRPV3 is known to lead to a significant inflammatory response in epidermal keratinocytes, and its increased expression and abnormal activity were shown to play a role in the development of AD-associated inflammatory processes. Thus, in order to elucidate the role of TRPV3 on sebocytes, we also examined the effect of TRPV3 activation on the immune phenotype of SZ95 sebocytes. Our results showed that the TRPV3 agonist carvacrol (500 μM) up-regulated the expression of several inflammatory cytokines after 6 h of treatment, whereas, interestingly, 2-APB (150 μM) proved ineffective.

The induction of an inflammatory response by carvacrol is largely TRPV3-dependent

To determine whether the inflammatory response induced by carvacrol was mediated by TRPV3, we repeated our experiments on TRPV3-silenced SZ95 sebocytes. We found that selective gene silencing of TRPV3 could significantly decrease carvacrol-induced up-regulation of IL-1β, IL-6, IL-8, and TNF-α, supporting that TRPV3 is involved in mediating pro-inflammatory effects of carvacrol. Interestingly, however, gene silencing did not affect the carvacrol-induced up-regulation of IL-1α.

Selective gene silencing of TRPV3 significantly reduces carvacrol-induced release of several inflammatory cytokines

Finally, to increase the translational relevance of our results, we also investigated how carvacrol affects the release of pro-inflammatory cytokines. In line with our results obtained at the mRNA level, we found that selective gene silencing of TRPV3 did not influence IL-1α levels in the cell supernatant, but significantly reduced the release of IL-1β, IL-6 and IL-8, while TNF-α concentrations were below detection threshold (unpublished observation). These findings suggest that TRPV3 indeed plays an important (but most likely not exclusive) role in mediating the pro-inflammatory effect of carvacrol.

Up to 14 μM FX can be used on human keratinocytes without risk of cytotoxicity

As a first step in our experiments, we wanted to determine the highest FX concentration that would not have an adverse effect on keratinocyte viability during 24 hours of treatment. Keratinocytes were tested with different FX concentrations (up to 100 μM). The corresponding curve fitting showed that the semi-cytotoxic concentration of FX over 24 h of treatment was ~25.43 μM, while the highest concentration that did not reduce the signal intensity below the vehicle control level was 14 μM.

Up to 14 μM FX does not reduce cell count of HaCaT keratinocyte during 24-hour treatments, whereas at 100 μM it has a cytotoxic effect

Subsequently, in order to exclude the possibility that FX at 14 μM might induce a cytotoxic effect, we also investigated the effect on cell number using another method, namely CyQUANT assay. This assay confirmed that, up to 14 μM, FX did not affect cell count over the course of 24-hour treatments compared to the vehicle-treated control group, whereas it significantly reduced it at 100 μM. Thus, we conclude that FX can be used at 14 μM concentration in our planned short (3 hours) and medium-term (24 hours) experiments without the risk of obvious of cytotoxicity.

FX significantly reduces p(I:C)-induced up-regulation of several pro-inflammatory cytokines in human epidermal keratinocytes

Next, we investigated the putative anti-inflammatory effect of FX in a previously optimized inflammatory model system. Using the TLR3 activator p(I:C) at a concentration of 20 μg/ml, we found that, as expected, p(I:C) significantly increased the mRNA expression of several inflammatory cytokines (IL-1α, IL-1β, IL-6 and IL-8) over the course of 3-hr treatments. These effects could be significantly reduced by concomitant application of FX (14 μM) in case

of IL-1α, IL-1β, and IL-8, whereas in case of IL-6, the alteration did not reach statistical significance (*P*=0.1615).

FX attenuates the effect of p(I:C) on the release of inflammatory mediators

Since our gene expression experiments suggested that the tested concentration of FX could effectively attenuate the p(I:C)-induced inflammatory response, we next proceeded to investigate this phenomenon at the protein level. In doing so, we first performed a cytokine array. The results of the array showed that 3-hr treatment with 20 μg/ml p(I:C) increased the release of 13 molecules and decreased the release of 4 molecules (a change of ≥1.5× was considered relevant alteration), and co-administration of FX (14 μM) attenuated the alterations in all cases. This suggested that the observed anti-inflammatory effect of FX at the mRNA level may also translate to the level of cytokine/chemokine release.

FX significantly reduces p(I:C)-induced IL-8 release (24-hr treatments)

To confirm these results, we further investigated the supernatants of the keratinocytes after 3 and 24 hours of treatments to monitor the early release of the already available cytokine pool induced by the inflammatory stimulus (3 h) and the secretion of *de novo* synthesized cytokines (24 h). In line with our Q-PCR data, we found that although FX appeared to reduce the p(I:C)-induced release of IL-6, the effect did not reach statistical significance at any of the time points; however, FX significantly reduced the release of IL-8 (24-hr treatments).

FX prevents p(I:C)-induced release of the itch-mediator endothelins in both immortalized (HaCaT) and primary human epidermal keratinocytes

Having shown that FX may exert an anti-inflammatory actions in our model system, we aimed to further investigate its putative beneficial effects. As mentioned above, pruritus is one of the most common symptoms of a wide range of skin conditions and epidermal keratinocytes play an important role in its development through the release of various pruritogens. Literature suggests that

various endothelins (and in particular endothelin-1) are key keratinocyte-derived mediators of pruritus. Moreover, the expression of endothelin-1 is up-regulated in the epidermis of lesional skin areas of AD patients and its plasma levels were not only elevated during disease exacerbation in AD but also showed a positive correlation with the severity of the symptoms. Since TLR3 has recently been shown to be up-regulated in several pruritic skin diseases and its activation by p(I:C) greatly enhanced endothelin release from primary human epidermal keratinocytes, we wanted to investigate how FX affects this process. In a preliminary experiment, using a pan-endothelin ELISA kit, we first showed that endothelin releases from HaCaT keratinocytes peaks after 24-hr treatments with 20 μg/ml p(I:C), similar to that of primary epidermal keratinocytes. Thus, in our subsequent experiments, we treated our cells by using 20 μg/ml p(I:C) for 24 hrs. Importantly, we found that the co-administration of FX (14 μM) almost completely suppressed p(I:C)-induced endothelin release in HaCaT keratinocytes as well as in primary human epidermal keratinocytes of five different donors. Our data thus suggest that FX may not only have anti-inflammatory effects but may also be able to alleviate certain types of pruritus, and these effects are likely to be donor-independent.

FX does not influence the p(I:C)-induced phosphorylation of p38 and IκBα, nor does it prevent the increase in mitochondrial production of reactive oxygen species (mtROS)

Stimulation of TLR3 can lead to activation of the NF-κB pathway via phosphorylation (and thus inactivation) of the inhibitory regulator IκBα, and TLR3 can also activate the p38 mitogen-activated protein kinase (MAPK) pathway. A previous experiment compared the effects of 10, 20, 30 and 60 min p(I:C) treatment (20 μg/ml). Our data indicated that p(I:C) induced phosphorylation of IκBα and p38 most strongly after 60 min (Western blot; *unpublished data*). Therefore, in our further experiments, we decided to use 60-

minute treatments. Our results demonstrated that co-administration of FX (14 μM) did not affect p(I:C)-induced phosphorylation of IκBα or p38 MAPK (Western blot), suggesting that the anti-inflammatory effect may occur without direct interaction with NF-κB or p38 MAPK activity.

In addition to its effects on the NF-κB and p38 MAPK pathways, TLR3 activation can also increase mitochondrial production of reactive oxygen species (mtROS). Although the co-administration of FX appeared to slightly reduce p(I:C)-induced mtROS production (MitoSOX Red labeling; 30 min treatments), the difference did not reach a statistically significant level (P=0.142).

The biological activity profile of 14 μM FX exhibits remarkable similarity to that of the PI3K inhibitor GDC0941

Having excluded the participation of three of the most obvious inflammatory signaling pathways in mediating the effects of FX, we intended to continue our experiments using the most unbiased assay possible. To this end, our collaborators turned to the BioMAP® Diversity PLUS® panel to explore the activity profiles of different concentrations of FX in 12 different human primary cell-based systems using predefined and optimized read-out parameters as endpoints and then compared the results to the profiles of other compounds in their database. This analysis showed that when FX was applied at a concentration of 14 μM, its activity profile showed remarkable similarity to that of the well-known inhibitor of the PI3K pathway, GDC0941 (also known as pictilisib) applied at a concentration of 370 nM.

It is noteworthy that although FX is known as a selective serotonin reuptake inhibitor, acting primarily by raising serotonin tone, serotonin was not among the top hits (in fact, when comparing 14 μM FX with serotonin at 90 μM, the Pearson correlation coefficient was only 0.354). This result suggested that at the anti-inflammatory concentrations we tested, FX is unlikely to act through the "classical" serotoninergic pathway. Said finding was also confirmed by cluster

analysis of the data. Indeed, as it became clear from the analysis, while the lower concentrations of FX (520 and 1,600 nM) clustered away from the higher FX doses (and at the same time closer to serotonin), the higher concentrations (4.7 and 14 μM) formed a separate cluster that showed a high similarity to the effects of different concentrations of GDC0941. All these results suggest that the anti-inflammatory and pruritogenic mediator release-suppressing effects of FX at 14 μM are probably not due to its known effects on its classical targets in serotoninergic signaling, but to direct or indirect inhibition of the pro-inflammatory PI3K signaling.

Importantly, PI3K is a central regulator of inflammatory processes. Some data suggest that FX may inhibit PI3K phosphorylation (and thus activation) *in vivo* in diabetic rats and mouse BV-2 microglia cells, while others have reported a concentration- and duration-dependent, indirect, biphasic effect on PI3K activity. Thus, the idea that changes in PI3K cascade activity may (also) be involved in mediating the biological effects of FX is not unprecedented in the literature.

GDC0941 mimics the anti-inflammatory and endothelin-release inhibitory effects of FX

Next, we wanted to investigate whether the beneficial effects of FX in epidermal keratinocytes can be mimicked by the inhibition of PI3K, and therefore we also tested the effect of the most similar molecule in terms of bioactivity profile, GDC0941, at the most appropriate concentration (370 nM) in our inflammatory model. We found that, when applied at a concentration of 370 nM, GDC0941 almost perfectly mimicked the biological effects of FX in human keratinocytes. Similar to FX (14 μM), GDC0941 inhibited p(I:C)-induced increased expression of IL-1α, IL-1β and IL-8, but (again similar to FX) had no significant effect on IL-6 mRNA expression. Moreover, it was also effective in reducing p(I:C)-induced release of IL-6, IL-8 and endothelins over the course of

24-hr treatments. These data suggest that the beneficial anti-inflammatory and pruritogen-release inhibitory effects of FX applied at 14 μM may indeed be mediated by inhibition of the PI3K pathway on human epidermal keratinocytes.

In silico molecular docking data suggest that FX may be able to occupy the binding site of GDC0941 on PI3K

As mentioned above, a considerable body of evidence suggests that FX may be able to modulate the activity of the PI3K pathway. However, to the best of our knowledge, prior to our experiments, there was no literature data on whether FX exerts this effect directly (i.e., by binding to PI3K enzymes) or indirectly (i.e., by interfering with PI3K-coupled signaling). Importantly, the remarkable similarities between the activity profiles of GDC0941 and FX, together with the effects of GDC0941 on cytokine and endothelin production by human keratinocytes raised the possibility that, just like GDC0941, FX may also directly inhibit PI3K if applied at sufficiently high concentrations. To investigate this putative direct interaction, our collaborator first performed an *in silico* molecular docking analysis to see whether, similar to GDC0941, FX might also be able to bind to the ATP-binding site of PI3K.

The properly optimized docking protocol consistently reproduced the experimentally determined position of the GDC0941 binding, as the best-estimated position differed by only 0.81 Å from the experimentally observed position. The modeling also succeeded in reproducing the key hydrogen bonds that mediate binding between GDC0941 and the corresponding side chains of the enzyme (D841, Y867, V882 and K802). By investigating the possible binding of FX, the analysis revealed that FX may be able to bind to the ATP-binding pocket of the PI3K enzyme in a very similar manner to GDC0941 and form hydrogen bonds with two side chains (D841 and Y867) to which GDC0941 also binds. In addition, molecular modeling shows that the benzene and phenyl groups of FX (trifluoromethyl) partially fill the hydrophobic site defined by the side chains

W812, I831, I881, F961 and I963, which is experimentally occupied by the hydrophobic thienopyrimidine core of GDC0941 during binding. While the above suggests that FX is indeed very likely able to occupy the binding site of GDC0941 on the PI3K enzyme, it is important to note that its binding energy determined by modeling was lower than that of GDC0941 (FX: -8.05 kcal/mol; GDC0941: -10.62 kcal/mol). This difference is most likely due to the smaller size of FX.

FX exerts less inhibition on PI3K activity in a cell-free enzyme activity assay

Next, we investigated whether FX can directly inhibit PI3K activity in a cell-free assay. Although high concentrations of FX were able to significantly inhibit PI3K activity, the observed inhibition was much weaker than that observed with GDC0941. Thus, our data suggest that although at sufficiently high concentrations FX can indeed directly inhibit PI3K activity, its anti-inflammatory effect is likely to be mediated through indirect interference with the PI3K-signaling and not via direct inhibition of the PI3K enzyme in human keratinocytes.

FX profoundly modulates the gene expression pattern induced by p(I:C)

Finally, to obtain a deeper insight into the nature of the anti-inflammatory effect of FX on human keratinocytes, RNA samples were collected after 24 h treatments with p(I:C) (20 μg/ml), p(I:C)+FX (14 μM) or appropriate vehicles. Principal component analysis showed that co-treatment with FX resulted in a distinct cluster compared to p(I:C)-treated cultures, suggesting that FX may have a significant effect on the p(I:C)-induced transcriptional response of human keratinocytes. In agreement with these results, pathway analysis of the genes that were significantly up-regulated by p(I:C) (>1.5-fold change; $P<0.05$; p(I:C) vs. control) using g:Profiler toolset showed that, as expected, p(I:C) treatment led to enrichment of inflammation-related GO:molecular function terms (e.g., “cytokine activity”, “cytokine receptor binding”, “chemokine activity”, “CXCR chemokine receptor binding” or “chemokine receptor binding”). Importantly, the same analysis using of genes that were significantly (>1.5-fold change; $P<0.05$) down-

regulated by FX (p(I:C)+FX vs. p(I:C)) yielded very similar results ("CXCR chemokine receptor binding", "chemokine activity" or "chemokine receptor binding"), suggesting that FX may indeed be able to attenuate the inflammatory effect of p(I:C).

Furthermore, careful analysis of the differentially expressed genes[*] showed that FX was able to prevent p(I:C)-induced up-regulation of several key inflammatory molecules and potential pruritus mediators. Without being exhaustive, the simultaneous administration of FX reduced expression of IL-6, IL-8 (CXCL8), GROα (also known as CXCL1), CXCL5 (also known as ENA-78), interferon alpha inducible protein 6 (IFI6, also known as G1P3), osteoprotegerin (TNFRSF11B), and interleukin-1 receptor type 2 (IL1R2), and up-regulated the rather immunosuppressive CXCL17. Taken together, these data strongly suggest that FX may exert significant anti-inflammatory effects in human epidermal keratinocytes.

[*] *The list of all differentially expressed genes can be found in the Supplementary data of the publication on which this dissertation is based; the raw data used to generate the lists are freely searchable in the NCBI SRA database (http://www.ncbi.nlm.nih.gov/bioproject/1022043).*

Discussion

In our previous studies, our team has demonstrated that TRPV3 is an important negative regulator of human hair growth, and we have also shown that TRPV3 activation induces an inflammatory response in human epidermal keratinocytes, while others have reported its role in dry skin-associated itch. The observed inflammatory symptoms and disruption of the lipid barrier suggested that inflammatory skin diseases induced by TRPV3 overexpression may involve skin cells other than keratinocytes that express TRPV3. Thus, in the first study that formed the basis of this thesis, we have investigated the expression and role of TRPV3 on professional lipid-producing cells of the skin, i.e., the sebocytes.

Immunohistochemical staining revealed that, like epidermal keratinocytes, TRPV3 is expressed in human sebaceous glands, and peripheral undifferentiated cells show stronger immunopositivity than centrally located terminally differentiated cells. We have also shown that TRPV3 is expressed at protein and mRNA levels in human sebaceous gland-derived SZ95 sebocytes. We also observed (consistent with our immunohistochemistry results) that TRPV3 expression is reduced in post-confluent, more differentiated cultures compared to highly proliferating pre-confluent cultures.

Functional investigation of the role of TRPV3 revealed that the synthetic TRPV3 activator 2-APB and the plant-derived carvacrol induced a significant increase in intracellular Ca^{2+} levels in human sebocytes. The elicited Ca^{2+} signals were almost completely abolished in the presence of the general TRP channel blocker ruthenium red but were unaffected by either AMG9810 or HC067047, which are selective antagonists of TRPV1 and TRPV4 channels, respectively, also expressed by the sebocytes and showing a high structural similarity to TRPV3. These results suggested that TRPV3 is expressed in a functionally active form in human sebocytes, however, as no specific TRPV3 activators and antagonists were commercially available, we decided to investigate the effect of reducing TRPV3 expression by siRNA transfection. Transfection of sebocytes resulted in a partial

but significant reduction in channel expression compared to "scrambled" RNA-transfected (control) cells, and significantly reduced the amplitude and rate of increase of agonist-induced Ca^{2+}- signals. These results provided convincing evidence that the activators used do indeed activate TRPV3 in human sebocytes.

Next, we assessed biological effects of the activators. We found that higher concentrations (2-APB: 200 μM; carvacrol: 1000 μM) significantly reduced the number of living cells in 24 hours, but lower concentrations (2-APB: 150 μM; carvacrol: 500 μM), which were still able to induce Ca^{2+} signals, did not affect the viability of sebocytes.

Since sebaceous lipids are essential for epidermal barrier function and, our group has previously shown that TRPV1 and TRPV4, which are structurally very similar to TRPV3, are potent negative regulators of sebaceous lipid production. Thus, we next investigated the effect of TRPV3 on lipid synthesis. Activation of TRPV3 with non-cytotoxic concentrations of 2-APB and carvacrol significantly reduced lipogenesis during AA-induced differentiation in scrambled RNA-transfected cells used as controls. However, the effect of TRPV3 agonists was significantly reduced in those sebocytes that were transfected with TRPV3-specific siRNA. We also observed that lipogenic effect of AA was slightly, but significantly stronger in TRPV3-silenced cells, suggesting that basal, homeostatic TRPV3 activity may decrease AA-induced sebaceous lipogenesis. Importantly, the effect of TRPV3 agonists was not limited to AA-induced lipid synthesis. The agonists also suppressed the lipogenic effects of the endocannabinoid anandamide and the combination of linoleic acid and testosterone, and slightly reduced basal lipid synthesis in untransfected cells as well. All these data suggest that TRPV3-mediated Ca^{2+} influx (similar to other interventions that increase intracellular Ca^{2+} concentration) exerts lipostatic effect in human sebocytes.

Interestingly, further investigating this phenomenon by flow cytometry, we found that carvacrol did not affect cell granularity (a flow cytometry read-out that usually correlates with differentiation of sebocytes), neither under control

conditions, nor after AA treatment, but selectively inhibited AA-induced lipid accumulation. Moreover, the latter effect was found to be TRPV-mediated, since it could be prevented by ruthenium red. Finally, we also demonstrated that carvacrol was able to down-regulate the expression of two important positive regulators of sebaceous lipid production, namely PPARγ and NRIP1, suggesting that its effect may be (at least partly) coupled to the down-regulation of these regulatory molecules.

In addition to lipid synthesis, sebocytes also play an important role in the regulation of immunological processes in the skin, and TRPV3 is known to significantly increase the production and release of pro-inflammatory cytokines in epidermal keratinocytes. Therefore, as a final step in our experiments exploring the effects of TRPV3, we investigated how TRPV3 activation affects the expression and release of specific pro-inflammatory cytokines. Our results clearly showed that the TRPV3 agonist carvacrol significantly enhanced the transcription of several pro-inflammatory cytokines over the course of 6-hr treatments, while, interestingly, 2-ABP was proven to be ineffective. To confirm the TRPV3 specificity of carvacrol, the same experiment was performed on TRPV3-silenced SZ95 sebocytes and a significant (yet, not complete) reduction in the effect of the activator was observed when compared to cells transfected with scrambled RNA used as a control. These results suggest that, just like in case of epidermal keratinocytes, activation of TRPV3 can induce a pro-inflammatory response in human sebocytes as well.

Taken together, our results suggest that sebocytes may be involved in the pathogenesis of inflammatory skin diseases associated with TRPV3 hyperactivity and skin dryness. Our data indicate that TRPV3 is not only a previously unknown negative regulator of sebaceous lipid synthesis, but can also induce a marked inflammatory response in cells, and thus its pharmacological inhibition could (theoretically) be beneficial in the treatment of these skin diseases.

The focus of the second article on which this dissertation is based, is on a widely used antidepressant, FX, a member of the selective serotonin reuptake inhibitor family. FX is know to be a safe agent that was shown to exert anti-inflammatory, anti-pruritic, pro-wound healing, and anti-AD effects in certain models. Considering the above pieces of information, it is not surprising that several diseases and conditions have recently emerged as potential new indications for FX, and in the present study we aimed to further investigate its putative beneficial effects using human epidermal keratinocytes.

We found that a non-cytotoxic concentration of FX (14 μM) was able to significantly reduce the p(I:C)-induced upregulation of several pro-inflammatory cytokines (IL-1α, IL-1β and IL-8), whereas the effect was not statistically significant for IL-6 (Q-PCR). In addition, FX significantly reduced the p(I:C)-induced release of IL-8 (ELISA; 24-hr treatments), a key regulator of skin inflammatory processes that is also used as a biomarker to monitor therapeutic efficiency in AD.

Pruritus is a leading symptom of many inflammatory skin diseases, including AD, and the "itch-scratch cycle" is known to contribute to the pathogenesis of these diseases. Thus, we next wanted to extend our studies to the keratinocyte-derived pruritogen endothelins. Endothelin-1 is known to be elevated not only in the lesional epidermis of AD patients, but its plasma levels also correlated with severity of the symptoms. It is important to note that the release of endothelin is (among other things) regulated by TLR3 signaling. Via the activation of TLR3, p(I:C) was found to increase endothelin-1 release from primary human epidermal keratinocytes, and TLR3 expression was significantly increased in the lesional epidermis of several pruritic skin diseases (i.e., AD, prurigo nodularis, and psoriasis). Since p(I:C) treatment also increased TLR3 expression in human epidermal keratinocytes, it is hypothesized that TLR3, as well as keratinocyte-derived endothelin release induced by the pathological TLR3

signaling, may contribute significantly to the development of the itch-scratch cycle.

It is important to note here that the available scant evidence suggests that the concentration of FX in the skin may be less than half (about 41%) of its plasma concentration. Thus, considering the usual plasma levels of FX (i.e., ~0.4-2 μM), it seems highly unlikely that a sufficiently high concentration in the skin can be achieved when administered systemically at non-cytotoxic doses. In contrast, a concentration of 14 μM would be readily achievable using appropriate topical formulations. Such limited, local application of FX would probably not lead to relevant systemic FX exposure or to systemic (side) effects.

Next, we wanted to explore the mechanism of the aforementioned beneficial effects. It is worth mentioning here that the test concentration of FX used in our experiments (14 μM) is much higher than the K_i value (3 nM) shown for the serotonin transporter (SLC6A4) that it inhibits, and (as already discussed), significantly higher than the usual plasma levels (~0.4-2 μM) measured in patients treated with FX. Based on these and several other literature data, we concluded that the effects of FX on keratinocytes might be mediated by non-classical cellular target(s).

Having excluded three possible anti-inflammatory mechanisms (i.e., inhibition of p(I:C)-induced NF-κB and p38 MAPK activation, and reduction of mtROS production), we decided to adopt a completely unbiased approach to explore the mechanism of action. Our collaborators investigated the biological activity profile of FX in 12 human primary cell-based systems using the BioMAP® Diversity PLUS® panel. We found that, when FX was applied at a concentration of 14 μM, its activity profile largely overlapped with that of the well-known PI3K-inhibitor GDC0941 (also known as “pictilisib”; 370 nM) (Pearson’s correlation coefficient (r): 0.926). Interestingly, serotonin was not among the top hits (Pearson’s correlation coefficient (r) was only 0.354 between serotonin at 90 μM and FX at 14 μM), suggesting that the effects of FX at 14 μM

were probably independent of “classical” serotoninergic signaling, and were rather mediated via the direct or indirect inhibition of pro-inflammatory PI3K signaling.

To challenge this hypothesis, we repeated our experiments using the aforementioned PI3K-inhibitor (GDC0941; 370 nM). We found that GDC0941 almost perfectly mimicked the biological effects of FX, namely, it significantly reduced the p(I:C)-induced up-regulation of IL-1α, IL-1β, and IL-8, while (similar to FX) it had no significant effect on the expression of IL-6 (Q-PCR). In addition, GDC0941 was effective in reducing p(I:C)-induced IL-8 and endothelin release (ELISA). Interestingly, a small difference was also observed compared to FX, namely, unlike FX, GDC0941 significantly reduced p(I:C)-induced IL-6 release (ELISA), whereas this effect did not reach statistical significance in case of FX.

Despite the aforementioned subtle difference, our results suggest that FX may inhibit the PI3K pathway in human keratinocytes. Although the concept that (at certain concentrations) FX can affect PI3K activity is not unprecedented in the literature, at the time of our experiments there were no published data available to indicate whether this occurs via direct, indirect, or combined (i.e., direct and indirect) inhibition of PI3K.

To address this question, using *in silico* molecular modelling, our collaborator first determined that FX may be able to occupy the same binding site on PI3K as GDC0941. Next, encouraged by the above *in silico* data, we used a cell-free activity assay to test whether FX is able to directly inhibit PI3K activity. In a perfect agreement with our *in silico* data, we found that a sufficiently high concentration of FX was indeed able to significantly inhibit PI3K activity; however, its effect was much weaker compared to GDC0941. Our data therefore suggest that, although its effect may be complemented by direct enzyme inhibition, FX is likely to indirectly inhibit the PI3K-coupled pro-inflammatory signaling pathway in human epidermal keratinocytes. Nevertheless, our results suggest that appropriate chemical modification of the FX scaffold to target

additional residues at the ATP-binding site of PI3K may lead to novel compounds with improved potency and efficacy against PI3K. Hence, our present results may also provide a starting point for future intelligent drug design studies leading to the development of novel FX derivatives with enhanced efficacy and potency towards PI3K.

In the final phase of our experiments, we performed RNA-Seq analysis to gain a deeper understanding of the biological effects induced by FX. Principal component analysis, pathway analysis, as well as individual assessment of the differentially expressed genes demonstrated that FX substantially influenced the pro-inflammatory effects of p(I:C) in human keratinocytes. Without being exhaustive, FX decreased the p(I:C)-induced up-regulation of the neutrophil chemoattractant chemokine GROα (also known as CXCL1) and CXCL5 (also known as ENA-78). At this point, it is worth noting that, according to some data, GROα (CXCL1) may also act as an itch mediator, and our data obtained with cytokine arrays suggest that FX may be able to suppress not only its expression but also its release.

In addition, FX significantly decreased the p(I:C)-induced up-regulation of IFI6 (G1P3), which was found to be up-regulated in both lesional and non-lesional skin of psoriasis patients. Moreover, FX down-regulated osteoprotegerin (TNFRSF11B), which is involved in the regulation of the RANKL - RANK interaction. Since epidermally expressed RANKL has been shown to induce immunosuppressive effects through increasing the number of regulatory T cells, downregulation of osteoprotegerin (TNFRSF11B) expression *in vivo* may add an additional layer to the anti-inflammatory effects of FX. Similarly, FX reduced p(I:C)-induced up-regulation of IL-1α and IL-1β receptor IL1R2. Since high levels of IL1R2 are thought to play a role in impaired Langerhans cell migration in aging skin, normalizing its expression may help to restore physiological cutaneous immune surveillance.

Furthermore, it is noteworthy that while p(I:C) down-regulated CXCL17, co-administration of FX significantly reduced this effect. CXCL17 has recently been found to attenuate skin inflammation via recruitment of myeloid-derived suppressor cells and regulatory T cells in an imiquimod-induced mouse model of psoriasis. Of course, it is to be determined in targeted future studies whether FX-induced CXCL17 up-regulation also manifests at the level of released cytokines, and whether this is limited to the p(I:C)-induced inflammatory responses, or is rather part of a more general anti-inflammatory effect. Either way, the FX-induced release and up-regulation of CXCL17 on a longer (>24 h) time scale may further enhance the *in vivo* anti-inflammatory effect of FX.

Taken together, our data clearly demonstrate that FX can exert a significant anti-inflammatory effect, and that its use may also lead to beneficial anti-pruritic effects by reducing the release of endogenous itch mediators such as endothelins (and perhaps others, e.g., GROα [CXCL1]). These effects are likely to be mediated through indirect inhibition of the pro-inflammatory PI3K pathway. In view of the well-established safety of FX, our data indicate that it would be worthwhile to clarify in targeted clinical trials whether the beneficial effects described above translate to beneficial clinical actions in inflammatory and pruritic skin diseases, when applied in appropriate topical formulations achieving sufficiently high cutaneous concentrations.

Summary

Our results show that TRPV3 is expressed in a functionally active form on human sebocytes, where its activation inhibits differentiation as well as sebaceous lipid production of human sebocytes induced by various lipogenic agents. Moreover, it significantly enhances the production and release of several pro-inflammatory cytokines. Our results suggest that the abnormal activity of TRPV3 may play a role in the pathogenesis of various inflammatory cutaneous conditions associated with skin dryness, and raise the possibility that TRPV3 antagonists may become effective therapeutic tools in the treatment of such diseases.

Our results of our experiments investigating the effects of fluoxetine showed that this molecule may exert significant anti-inflammatory effects on human epidermal keratinocytes, and it effectively reduces the release of the endogenous itch-mediator endothelins. Assessment of the biological activity profile of fluoxetine, RNA-Seq analysis, *in silico* binding assay, and direct enzyme activity measurements indicate that the effects are likely to be mediated through indirect inhibition of the pro-inflammatory PI3K pathway. Considering the well-established safety profile of FX, our findings highlight the possibility of a dermatological repurposing of FX, but, of course, further clinical studies are required to investigate whether the beneficial effects demonstrated in this study also translate *in vivo* when applied in appropriate topical formulations in dermatological conditions associated with inflammation and pruritus.

Acknowledgments

I would like to take this opportunity to thank all those who contributed in any way to the realization of this thesis, either by providing human and financial support or by their professional advice and assistance.

First of all, I would like to thank Prof. Dr. László Csernoch, head of the Department of Physiology, who provided the necessary conditions for my Ph.D. thesis. I thank my former (Prof. Dr. Tamás Bíró) and current (Dr. habil. Attila Oláh) Ph.D. supervisors for their trust in me, for their professional support over the years and for being an important example for me from a professional and human point of view.

I would like to thank the staff, past and present Ph.D. and undergraduate students of the laboratory and the department, who have always selflessly come to my aid when I needed. Among them, I am extremely grateful for the help of my direct colleagues, namely Dr. Gabriella Czifra, Dr. István Balázs Tóth, Dr. Attila Gábor Szöllősi, Dr. Magdolna Szántó, Dr. Johanna Mihály, Dr. Gabriella Béke, Dr. Arnold Markovics, Dorottya Ádám, József Arany, Dr. Shahrzad Alimohammadi, Dr. Balázs Kelemen, Dr. Erika Lisztes, Nikolett Fricz, Anita Galicz, Erika Hollósi, Judit Szabó-Papp, Gergő Elek Kovács, and Mónika Barotáné Kovács.

I am grateful for the work of my supervised undergraduate students, Petra Faragó and Dr. Fruzsina Sárkány. They are very talented, committed and hard-working students; it is always a great pleasure for me to think about our work together.

I thank our collaborative partners (Prof. Dr. Christos C. Zouboulis, Prof. Dr. Christoph Abels, Dr. Ágnes Pór, Dr. Orsolya Matolay and many others) for their invaluable help.

Finally, in these few lines, I would like to thank my Parents, Husband, Brother, Grandmother,Godfather and my Mother-in-Law; without them I would not have made it this far, I am very grateful to them. I would especially like to

thank Zsófia Pénzes for the friendship we made during my undergraduate studies in biology and for her professional and spiritual support during my Ph.D. years.

Preparation of the Ph.D. thesis was supported by multiple research grants and scholarships, namely, GINOP-2.3.2-15-2016-00015 (I-KOM), EFOP-3.6.3-VEKOP-16-2017-00009, the National Talent Programme of the Human Resources Funding Agency (ID: NTP-NFTÖ-18-B-0168), the New National Excellence Programme of the Ministry for Innovation and Technology (ID: ÚNKP-19-3-I-DE-141), and “FK_20” (IDs: 125055, 134235) and “K_2016” (ID: 120552) grants of the National Research, Development and Innovation Office. Besides the above financial resources, investigation of the effects of fluoxetine involved support from our industrial collaborative partner (Dr. August Wolff GmbH & Co. KG Arzneimittel; Bielefeld, Germany) as well.

Appendix - List of own publications

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/527/2023.PL
Subject: PhD Publication List

Candidate: Kinga Fanni Volascsekné Tóth
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Tóth, K. F.**, Ádám, D., Arany, J., Ramirez, Y. A., Bíró, T., Drake, J. I., O'Mahony, A., Szöllősi, A. G., Póliska, S., Kilic, A., Soeberdt, M., Abels, C., Oláh, A.: Fluoxetine exerts anti-inflammatory effects on human epidermal keratinocytes and suppresses their endothelin release.
   Exp. Dermatol. "Accepted by Publisher", 2023.
   IF: 3.6 (2022)

2. Szántó, M., Oláh, A., Szöllősi, A. G., **Tóth, K. F.**, Páyer, E., Czakó, N., Pór, Á., Kovács, I., Zouboulis, C. C., Kemény, L. V., Bíró, T., Tóth, I. B.: Activation of TRPV3 inhibits lipogenesis and stimulates production of inflammatory mediators in human sebocytes: a putative contributor to dry skin dermatoses.
   J. Invest. Dermatol. 139 (1), 250-253, 2019.
   DOI: http://dx.doi.org/10.1016/j.jid.2018.07.015
   IF: 7.143

List of other publications

3. Ádám, D., Arany, J., **Tóth, K. F.**, Tóth, I. B., Szöllősi, A. G., Oláh, A.: Opioidergic Signaling: a Neglected, Yet Potentially Important Player in Atopic Dermatitis.
   Int. J. Mol. Sci. 23 (8), 4140, 2022.
   DOI: http://dx.doi.org/10.3390/ijms23084140
   IF: 5.6

4. Angyal, Á., Pénzes, Z., Alimohammadi, S., Horváth, D., Takács, L., Vereb, G., Zsebik, B., Bíró, T., **Tóth, K. F.**, Lisztes, E., Tóth, I. B., Oláh, A., Szöllősi, A. G.: Anandamide Concentration-Dependently Modulates Toll-Like Receptor 3 Agonism or UVB-Induced Inflammatory Response of Human Corneal Epithelial Cells.
   Int. J. Mol. Sci. 22 (15), 7776, 2021.
   DOI: http://dx.doi.org/10.3390/ijms22157776
   IF: 6.208

5. Markovics, A., Angyal, Á., **Tóth, K. F.**, Ádám, D., Pénzes, Z., Magi, J., Pór, Á., Kovács, I., Töröcsik, D., Zouboulis, C. C., Bíró, T., Oláh, A.: GPR119 is a potent regulator of human sebocyte biology.
J. Invest. Dermatol. 140 (10), 1909-1918, 2020.
DOI: https://doi.org/10.1016/j.jid.2020.02.011
IF: 8.551

6. Szabó, I. L., Lisztes, E., Béke, G., **Tóth, K. F.**, Paus, R., Oláh, A., Bíró, T.: The phytocannabinoid (-)-cannabidiol (CBD) operates as a complex, differential modulator of human hair growth: anti-inflammatory submicromolar versus hair growth inhibitory micromolar effects.
J. Invest. Dermatol. 140 (2), 484-488, 2020.
DOI: http://dx.doi.org/10.1016/j.jid.2019.07.690
IF: 8.551

7. **Tóth, K. F.**, Ádám, D., Bíró, T., Oláh, A.: Cannabinoid signaling in the skin: therapeutic potential of the "c(ut)annabinoid" system.
Molecules. 24 (5), 918, 2019.
DOI: http://dx.doi.org/10.3390/molecules24050918
IF: 3.267

8. Markovics, A., **Tóth, K. F.**, Sós, K., Magi, J., Gyöngyösi, A., Benyó, Z., Zouboulis, C. C., Bíró, T., Oláh, A.: Nicotinic acid suppresses sebaceous lipogenesis of human sebocytes via activating hydroxycarboxylic acid receptor 2 (HCA2).
J. Cell. Mol. Med. 23 (9), 6203-6214, 2019.
DOI: http://dx.doi.org/10.1111/jcmm.14505
IF: 4.486

Total IF of journals (all publications): 47,406
Total IF of journals (publications related to the dissertation): 10,743

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

29 November, 2023