ASSESMENT OF CUTANEOUS ANTI-INFLAMMATORY EFFECTS OF PHYTOCANNABINOIDS

Final Report

Introduction:

The skin is the largest organ of the human organism and it serves as a protective shield, being a physical-chemical barrier and immune barrier against pathogen microorganisms. If the immunological barrier is impaired, certain inflammatory skin disorders develop (such as e.g., atopic dermatitis,), which impair the quality of life of millions worldwide. Despite extensive efforts, we still lack easily applicable, well-tolerated, and universally effective tools to manage these conditions. Hence, there is an emerging demand from both the medical community and the society to identify new therapeutic tools and targets. Epidermal keratinocytes play a crucial role in skin homeostasis; therefore some mediators of keratinocyte origin play an important role in the pathogenesis or several skin diseases. In our project we aimed at defining and exploiting the anti-inflammatory potential of selected *Cannabis sativa*-derived cannabinoids ("phytocannabinoids"), their fluorinated derivatives, respectively β-caryophyllene (BCP) which is a natural bicyclic sesquiterpene also found in *Cannabis sativa*. Moreover, during the course of our experiments,we expanded our studies to another naturally occurring component, the sodium salt of N, N-dimethylglycine (DMG-Na), which is part of the endogenous homocysteine pathway and is found in several food sources and plants including *Cannabis sativa*.

Results:

1. In the first year in the context of Task 1, different concentrations of plant-derived and fluorinated phytocannabinoids were investigated on the viability of human immortalized keratinocytes (HaCaT and HPV-KER) by colorimetric MTT assays after 24-48-72 hours treatment. According to our results, the investigated compounds (CBD, HUF101, HUF103 and HU559a) impaired the cell viability of HPV-KER at 10 μM concentrations; therefore for our further experiments we only used non-toxic concentrations.

In the next part of our experiments, we modelled certain dermatological disordersin vitro (non-specific, allergic contact dermatitis, chemical irritant caused dermatitis, solar, atopic, bacterial, viral and other microbial origin dermatitis). e investigated the potential anti-inflammatory effects of cannabidiol (CBD) and other semi-synthetic cannabinoid derivatives (HUF101, HUF103 and HU559a) in thesemodels of pro-inflammatory skin diseases where inflammation was induced by different pro-inflammatory agents (such as sodium-dodecyl-sulphate, nickel, carvacrol, SLIGRL, UVB irradiation, poly(I:C), Staphyloccus entrotoxin – thymic stromal lymphopoietin).

After the administration of the above mentioned pro-inflammatory inducers we observed a significant up-regulation of the gene expressions of several inflammatory cytokines compared to the control groups, such as interleukin-(IL)-1α, IL-1β, IL-6, IL-8, respectively tumor necrosis factor (TNF)-α. Importantly, these pro-inflammatory effects were significantly counteracted upon the application of CBD and fluorinated semi-synthetic phytocannabinoids

in almost all of the investigated models. Further, we also observed that the fluorinated compounds exerted a much more efficient anti-inflammatory effect when compared to CBD.

	CBD			HUF101			HUF103			HU559a		
Conc.	0.01 µM	0.1 µM	1 µM	0.01 µM	0.1 µM	1 µM	0.01 µM	0.1 µM	1 µM	0.01 µM	0.1 µM	1 µM
MODEL 1 (SDS)												
IL6	69%	67%	61%	114%	10%	114%	76%	19%	146%	543 %	76%	47%
IL8	30%	39%	57%	26%	5%	84%	7%	6%	101%	251%	31%	26%
MODEL 2 (Ni)												
IL6	39%	24%	57%	22%	64%	30%	1%	24%	3%	1%	27%	16%
IL8	34%	15%	73%	10%	10%	15%	1%	84%	1%	1%	23%	32%
MODEL 3 (Carv)												
IL1α	150%	119%	151%	100%	100%	113%	7%	108%	2%	2%	111%	159%
TNFα	50%	95%	156%	104%	130%	117%	43%	108%	770%	2%	107%	116%
IL6	106%	98%	123%	56%	97%	147%	5%	100%	1%	4%	100%	181%
IL8	187%	161%	219%	144%	355%	677%	11%	161%	1%	5%	160%	280%
MODEL 4 (SLIGRL)												
IL6	131%	157%	173%	121%	362%	144%	0%	335%	16%	5%	141%	115%
IL8	137%	76%	124%	85%	218%	68%	0%	214%	14%	3%	108%	83%
MODEL 5 (UVB)												
IL1α	110%	222%	145%	102%	77%	157%	6%	85%	3%	4%	109%	191%
IL1β	109%	149%	141%	44%	62%	128%	5%	53%	26%	2%	123%	238%
IL6	89%	88%	75%	71%	92%	119%	7%	46%	2%	2%	60%	224%
IL8	85%	104%	85%	50%	75%	136%	4%	67%	1%	2%	48%	97%
MODEL 6 (pIC)												
IL1α	37%	77%	43%	29%	44%	16%	462%	15%	37%	465%	15%	44%
IL1β	86%	162%	413%	185%	210%	288%	10189%	142%	1001%	9126%	192%	445%
IL6	208%	110%	47%	27%	31%	31%	720%	13%	309%	748%	25%	111%
IL8	133%	90%	43%	24%	20%	34%	427%	17%	192%	644%	23%	60%
MODEL 7 (SEB-TSLP)												
IL1A	139%	134%	208%	77%	137%	258%	4%	68%	1%	13%	58%	67%
IL6	71%	60%	95%	66%	88%	41%	3%	22%	2%	4%	25%	24%
IL8	60%	40%	22%	62%	76%	23%	3%	33%	1%	9%	42%	25%

mRNA expression decreases < 30 % after CBD or F-CBD treatment compared to the expression of the inductor

mRNA expression decreases < 50 % after CBD or F-CBD treatment compared to the expression of the inductor

Table 1. mRNA levels expressed in percentages, 100% is the gene expression level observed after induction by the applies inflammatory agents as it follows: Model 1. ‘‘Non-specific’’ irritation/inflammation model – is performed by employing a detergent: SDS (sodium secondary dodecan sulphate) on HaCaT keratinocytes in order to mimic common irritative dermatitis. Model 2: ‘‘Contact’’ irritation/inflammation model - is performed by employing nickel on HPV-KER keratinocytes in order to mimic contact dermatitis. Model 3: ‘‘Chemical’’ irritation/inflammation model - is performed by plant-derived activators of TRPV 3 channel on HPV-KER keratinocytes in order to mimic chemical-induced irritative dermatitis. Model 4: ‘‘Protease’’ irritation/inflammation model - is performed by employing activators of PAR2 receptor and signaling on HaCaT keratinocytes in order to mimic pruritic dermatitis. Model 5: ‘‘UVB’’ irritation/inflammation model - is performed by employing UVB light on HPV-KER keratinocytes in order to mimic solar dermatitis. Model 6: ‘‘TLR’’ irritation/inflammation model - is is performed by employing activators of TLR signaling on HaCaT keratinocytes in order to mimic bacterial, viral, and other microbial dermatitis. Model 7: ‘‘AD-related’’ irritation/inflammation model – AD-cocktail (SEB and TSLP) is applied on HPV-KER keratinocytes to mimick atopic dermatitis.

We also examined the release of the inflammatory cytokines IL6 and IL8 on protein level by ELISA assays which further confirmed the results of the experiments obtained by QRT-PCR reflecting the robust anti-inflammatory effects of the cannabinoids.

The results of our experiments were presented on several conferences and workshops as it follows:

Posters:

[1] N. Miltner, **J. Mihály**, V. Tubak, R. Mechoulam, E. Russo, T, Bíró, Assessment of the anti-inflammatory effects of fluorinated semi-synthetic phytocannabinoids in human in vitro inflammatory keratinocyte model systems, 46th Annual Meeting of the Hungarian Immunological Society, Velence, Hungary, 18-20.10.2017

[2] **J. Mihály**, N. Miltner, V. Tubak, R. Mechoulam, E. Russo, T. Bíró, Anti-inflammatory effects of novel semi-synthetic phytocannabinoids in human in vitro inflammatory keratinocyte model systems, 16th EAACI

Immunology Winter School, Basic Research in Allergy and Clinical Immunology, Saas-Fee, Switzerland, 25-28.01.2018

Conference Presentations:

[3] **J. Mihály,** N. Miltner, V. Tubak, R. Mechoulam, E. Russo, T, Bíró, Assessment of the anti-inflammatory effects of fluorinated semi-synthetic phytocannabinoids in human in vitro inflammatory keratinocyte model systems, Barcelona, Spain, November 9-11, 2017

[4] N. Miltner, J. Mihály, V. Tubak, R. Mechoulam, E. Russo, T, Bíró Assessment of the anti-inflammatory effects of novel semi-synthetic phytocannabinoids in human in vitro pro-inflammatory keratinocyte model systems, EADV-ESDR Summer Research Workshop Advanced molecular biology tools in dermatological research, Naples, Italy, June 4-8, 2018

[5] **J. Mihaly,** N. Miltner, V. Tubak, R. Mechoulam, E. Russo, T, Bíró, Assessment of the anti-inflammatory effects of phytocannabinoids in skin, Praetor International Conference, Interdisciplinary Session, Debrecen, Hungary, October 11, 2018

2. During the second year we investigated the potential anti-inflammatory effects of β-caryophyllene (BCP) which was previously shown to exert beneficial effects in barious models of anxiety, depression, arthritis, pain, and inflammation. Further, it was found to have antimicrobial, antioxidant, neuroprotective and anti-cancer effects. Despite the extensive usage of BCP, its potential anti-inflammatory effects in human keratinocytes are still poorly investigated. Therefore, we also aimed to assess the potential cutaneous anti-inflammatory actions of BCP in four of our previously established in vitro human epidermal keratinocyte models; i.e., contact irritation, chemical irritation, UVB-induced, and atopic dermatitis-like model.

Similar to our findings with CBD and its derivatives, the upregulated expressions of certain pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, TNF-α), induced by the proinflammatory agents, were significantly down-regulated upon the administration of BCP in the chemical, UV-B-induced and atopic dermatitis-relevant irritation/inflammation models but not in the contact irritation/inflammation model.

These intriguing data, along with the aforementioned results with the phytocannabinoids, invite further pre-clinical and clinical studies to exploit their therapeutic potential in various cutaneous inflammatory conditions.

The results of our experiments with BCP were presented on conferences as it follows:

Posters:

[6] N. Miltner, **J. Mihály**, V. Tubak, R. Mechoulam, T. Bíró, Investigation of anti-inflammatory effect of β-caryophyllene in human in vitro inflammatory keratinocyte model systems, 91st Annual Meeting of the Hungarian Dermatological Society, Budapest, Hungary, 29-30.11.2018

[7] N. Miltner, **J. Mihály**, V. Tubak, R. Mechoulam, E. Russo, T. Bíró, Investigation of anti-inflammatory effect of β-caryophyllene in human in vitro inflammatory keratinocyte model systems, 13th EFIS-EJI Tatra Immunology Conference, Strbske Pleso, Slovakia, 9-13.06.2018

Abstract:

[8] N. Miltner, **J. Mihály**, V. Tubak, R. Mechoulam, T. Bíró, Investigation of anti-inflammatory effect of β-caryophyllene in human in vitro inflammatory keratinocyte model systems, Bőrgyógyászati és Venerológiai Szemle, 2018. 94/6, p 309 Meeting abstract: 12, Published: DECEMBER 2018

3. Also during the second year we performed the majority of the animal experiments. The goal of these experiments was to investigate the effects of the selected cannabinoid derivatives in 3 different murine inflammatory models; therefore we investigated the effects of the naturally occurring CBD and the fluorinated compound HUF101, at 1 and 10 µM concentrations.

During our experiments we used Balb/c mice (n=5/group), anesthesia was performed with 100 mg/kg ketamine and 5 mg/kg xylazin i.p. respectively.

We used the following in vivo inflammatory models:

a) Oxazolone-induced allergic contact dermatitis – Sensitization on 2 consecutive days by 2% oxazolone (50-50 µl) on the shaved abdomen. On day 6, right ears are smeared with 2% oxazolone dissolved in 96% ethanol: 15-15 µl solution is applied on each of the inner and outer surfaces. Ear edema, neutrophil myeloperoxidase (MPO) activity and body weight were measured. In this model, oxazolone increased the ear width with to about 120-130%, which was significantly reduced by CBD and HUF101, where the latter exhibited a more significant anti-inflammatory effect. Neutrophil granulocyte accumulation monitored by myeloperoxidase activity (MPO) increased significantly 24 hrs after elicitation of dermatitis. Significantly higher MPO activity was observed in CBD treated ears compared to the oxazolone-treated control. Vehicle treated ears also showed increased MPO activity.

b) Imiquimod-induced psoriasiform skin inflammation – 20 mg Aldara cream (inducer: 5% imiquimod) and 20 mg Vaseline (control) were applied to the shaved back skin of mice and treatments were repeated after 4 days. Dorsal skin edema, blood perfusion and bodyweight were investigated. In this model, the formed skin edema caused by imiquimod was not decreased neither by the 1 µM, nor by the 10 µM CBD. Neither 1 µM, nor 10 µM HUF101 was not effective in this experimental setup.

c) Irritant dermatitis– The mouse ear was smeared with 10 µl of 10 % v/v croton oil dissolved in acetone on one ear and acetone alone (control) on the other ear. Ear edema, neutrophil myeloperoxidase (MPO) activity and body weight were measured. Both the 1 µM CBD and the 10 µM CBD significantly reduced the ear edema caused by croton oil. Yet, we could not find significant differences in the MPO activity in the CBD treated groups compared to the control. Neither 1 µM, nor 10 µM HUF101 was not effective in the irritant dermatitis model.

As a summary of the animal experiments, we can conclude that the topically applied CBD, respectively fluorinated cannabinoid derivative HUF101 significantly reduced ear edema in 1 µM concentration in the oxazolone-induced allergic contact dermatitis mouse model. In the imiquimod-induced psoriasiform skin inflammation model no significant differences were observed, whereas in the irritant dermatitis mouse model CBD significantly reduced ear edema, but had no effect on the MPO activity.
The results of our experiments were presented on a world congress:

Poster:

[9] N. Miltner, G. Béke, Á. Angyal, Á. Kemény, E. Pintér, Zs. Helyes, T. Bíró, **J. Mihály**, Assessment of the anti-inflammatory effects of cannabidiol and its fluorinated derivative in in vitro and in vivo models of atopic dermatitis, International Investigative Dermatology 2018, Orlando, Florida, USA, 16-19.05.2018

Published abstract:

[10] N. Miltner, G. Béke, Á. Angyal, Á. Kemény, E. Pintér, Zs. Helyes, T. Bíró, **J. Mihály**, Assessment of the anti-inflammatory effects of cannabidiol and its fluorinated derivative in in vitro and in vivo models of atopic dermatitis JOURNAL OF INVESTIGATIVE DERMATOLOGY Volume: 138 Issue: 5 Supplement: S Pages: S173-S173 Meeting Abstract: 1020 Published: MAY 2018

4. As an extension of our in vitro experiments, we also tested the potential anti-inflammatory effects of CBD, the fluorinated compounds HUF101 and HUF103, respectively BCP on a lipopolysaccharide (LPS)-induced mouse macrophage RAW 264.7 cell model of inflammation was used. LPS-induced upregulated mRNA levels of Il-1α and Il-1β were reduced by BCP, CBD while this effect was further reduced by the F-CBDs in most of the applied concentrations. Further, the LPS-induced NF-κB activity was also reduced by all applied concentrations of CBD pre-treated for 30 min but not by BCP. Most importantly, whereas CBD exerted robus anti-inflammatory actions on RAW 264.7 macropahges, fluorinated semi-synthetic phytocannabinoids proved to be more effective than the non-fluorinated, plant-derived CBDs.

The results of these experiments were presented as it follows:
[11] N. Miltner, **J. Mihály**, R. Mechoulam, T. Bíró, Assessment of the potential anti-inflammatory effect of phytocannabinoids in an in vitro mouse inflammatory model system, 17th EAACI Immunology Winter School, Basic Research in Allergy and Clinical Immunology, Trysil, Norway, 24-27.01.2019

5. In the final years of the grant we aimed at completing Task 3 and Task 4. We performed FLIPR (Fluorescence Imaging Plate Reader)-based Ca-imaging experiments with the most promising flourinated phytocannabinoids using the Ca-sensitive Fluo-4 AM fluorescent dye, in order to observe whether these compounds are capable of increasing intracellular Ca levels. After the optimization of the Ca-imaging techniques first we examined the possible effects of CBD, HUF101 and HUF103 on human epidermal HaCaT keratinocytes and on human dermal fibroblasts, both in a normal Ca-containing and Ca-free environment. Interestingly, in contrast to the positive control, TRPV4 ion channel agonist GSK1016790A, neither compound modified the intracellular Ca concentration of these cells up to 100 µM concentration, suggesting that their versatile cellular and molecular effects are most probably not directly linked cellular Ca handling.

6. We also assessed the effects of various compounds in the ex vivo human full-thickness skin organ culture model. Full thickness punch biopsies were obtained from dermato-surgical surplus materialand were cultured at air-liquid interface in a well-defined culture medium. First, cellular inflammation was induced by a toll-like receptor (TLR) activating cocktail (i.e. 1 µg/ml LPS, 1 µg/ ml lipoteichoic acid and 1 µg/ml Polyinosinic:polycytidylic acid) and then the potential anti-inflammatory effects of CBD (3 and 30 µM), BCP (30 and 300 µM) and the fluorinated CBD compound HUF101 (3 and 30 µM) were assessed for 24-48-72 hours. By using quantitative histomorphometry on hematoxylin-eosin stained histological sections, we observed that in the TLR-treated groups the epidermis was thickened and was highly infiltrated by inflammatory cells. In the groups treated with the higher concentrations of CBD/BCP/HUF101, these symptoms were highly ameliorated. As a next step, RNA was isolated from the remaining samples and the gene expression profile of certain pro-inflammatory cytokines (IL6, IL8, OCLN, CLDN és MCP1) was determined. After 24 hours IL8 levels decreased significantly upon the administration of 30 µM CBD, 30-300 µM BCP and 3-30 µM HUF101, wheras after 48 hours no significant anti-inflammatory effects could be observed. After 48 hours IL6 levels decreased upon both CBD concentrations and the higher concentrations of BCP and HUF101. OCLN és CLDN gene expression decreased after the application of both concentraions of BCP. MCP1 gene expression significantly decreased after TLR cocktail-induced inflammation, except for the higher concentration of HUF101.

7. Finally we also performed experiments with the sodium salt of dimehtylglycine (DMG-Na), which is a non-cannabinoid, non-terpenoid compound found in most plants. DMG and its sodium salt may function as a source of glycine for glutathione synthesis, and hence may improve cellular and tissue antioxidant capacities. Indeed, in various animal studies that model pathologies of e.g. the gastrointestinal tract, liver, skeletal muscle and peripheral neurons, both DMG and its sodium salt were shown to reduce oxidative stress damage by exhibiting scavenger activities against excess free radicals and to improve the utilization of oxygen, hence they can be conductive to the body's redox status and its regeneration and repair. Moreover, DMG, acting as a methyl donor, has been suggested to improve immunity and enhance immune responses.

Despite these beneficial effects for maintaining the homeostasis of the body, to our knowledge, so far there is no report in the literature on the putative effects of DMG or its salt on the human skin, the largest organ of the body with constant cellular and tissue regeneration-rejuvenation-remodeling, and continuous exposition to stressor challenges from the environment. We found that N,N-Dimethylglycine sodium salt (DMG-Na) promoted the proliferation of cultured human epidermal HaCaT keratinocytes without compromising the cellular viability of these cells. In a scratch wound closure assay, DMG-Na augmented the rate of wound closure, demonstrating that it promotes keratinocyte migration. Further, DMG-Na treatment of the cells resulted in the upregulation of the synthesis and release of specific growth factors. Intriguingly, DMG-Na also exerted robust anti-inflammatory and anti-oxidant effects as assessed in three different models of human keratinocytes, mimicking microbial and allergic contact dermatitis as well as psoriasis and UVB irradiation induced solar dermatitis. These results identify DMG-Na as a highly promising novel active to promote epidermal proliferation, regeneration and repair, and to exert protective functions. Further pre-clinical and clinical studies are under investigation to prove the seminal impact of topically applied DMG-Na on relevant conditions of the skin and its appendages.

A manuscript summarizing these results was published recently:

[11] Lendvai A, Béke G, Hollósi E, Becker M, Völker JM, Schulze Zur Wiesche E, Bácsi A, Bíró T, **Mihály J.** N,N-Dimethylglycine Sodium Salt Exerts Marked Anti-Inflammatory Effects in Various Dermatitis Models and Activates Human Epidermal Keratinocytes by Increasing Proliferation, Migration, and Growth Factor Release. Int J Mol Sci. 2023 Jul 9;24(14):11264. doi: 10.3390/ijms241411264. PMID: 37511024; PMCID: PMC10379135.

Published abstract:

[12] Lendvai A, Béke G, Hollósi E, Becker M, Völker JM, Schulze Zur Wiesche E, Bácsi A, Bíró T, **Mihály J.** N,N-dimethylglycine sodium salt activates human epidermal keratinocytes and exerts anti-inflammatory effects in various dermatitis models, Immunológiai Szemle, 2023. October, XV/3

Poster:

[13] Lendvai A, Béke G, Hollósi E, Becker M, Völker JM, Schulze Zur Wiesche E, Bácsi A, Bíró T, **Mihály J.** N,N-dimethylglycine sodium salt activates human epidermal keratinocytes and exerts anti-inflammatory effects in various dermatitis models

Next, we aimed at investigating the effects of DMG-Na on the[Ca2+]i – eNOS – NO triad, which plays a crucial role in the microcirculation of the skin in in vitro cultures of primary human dermal microvascular endothelial cells (HDMECs). These in vitro data unambiguously showed that DMG-Na engages the "↑[Ca2+]i – eNOS activation – ↑NO synthesis" triad which, in turn, may induce endothelium-dependent vasodilation and hence boost skin microcirculation, upon e.g. topical application.

To probe this hypothesis, we designed a monocentric, single-blinded, randomized, **placebo-controlled** human *in vivo* study in which the effects of a topical applied 1% DMG-Na gel on skin blood flow and microcirculation (a key determinant of all cutaneous functions) were assessed. Notably, over the course of this *in vivo* study, although an increase in microcirculation was observed for all subjects, only one subject showed a very slight temporary red blotchiness of the skin about 60 minutes after application of the verum product. Otherwise, no adverse effects such as e.g., itching, stinging, feeling of tension, or any other discomfort were observed.

Taken together, these pioneer data provide the first human *in vivo* evidence that topical DMG-Na robustly increases the intensity of dermal blood flow by penetrating into deeper cutaneous layers.—Further, our complementary *in vitro* experiments clearly show that the above effects of DMG-Na are most probably due to its actions on HDMECs which in turn, induces endothelium-derived, NO-dependent vasodilation.

Thus, our in vivo and in vitro findings collectively suggest that DMG-Na is a highly promising novel active in conditions like e.g. skin aging, atrophy, acute or chronic wounds, hair loss of any kind and various dermatitis, in which the beneficial effects of DMG-Na to promote epidermal proliferation, regeneration and repair; to augment skin blood flow; and to exert protective functions could be of great impact. Further clinical studies are now warranted to explore the enormous application potential of DMG-Na in multiple human skin pathologies.

A manuscript summarizing these results is currently under preparation and will be submitted to Exp Dermatol. within a month:

[14] G. Béke, A. Lendvai, E. Hollósi, N. Braun, C. Theek, J. Kállai, Á. Lányi, M. Becker, J. M. Völker, E. Schulze zur Wiesche, A. Bácsi, T. Bíró, **J. Mihály**, N,N-Dimethylglycine Sodium Salt Enhances Human Skin Blood Flow by Inducing Endothelial Nitric Oxide Release. Exp. Dermatol. (under preparation).

Note: The grant was on hold from 01.04.2019 to 01.03.2021 due to maternal leave and I also received one year extension due to COVID-19 restrictions.