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Differential effectiveness of selected non-psychotropic phytocannabinoids on human sebocyte functions implicates their introduction in dry / seborrheic skin and acne treatment

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Differential effectiveness of selected non-psychotropic phytocannabinoids on human sebocyte functions implicates their introduction in dry/seborrheic skin and acne treatment

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ABSTRACT

Acne is a common skin disease characterized by elevated sebum production and inflammation of the sebaceous glands. We have previously shown that a non-psychotropic phytocannabinoid ((-)-cannabidiol [CBD]) exerted complex anti-acne effects by normalizing "pro-acne agents"-induced excessive sebaceous lipid production, reducing proliferation and alleviating inflammation in human SZ95 sebocytes. Therefore, in the current study we aimed to explore the putative anti-acne effects of further non-psychotropic phytocannabinoids ((-)-cannabichromene [CBC], (-)-cannabidivarin [CBDV], (-)-cannabigerol [CBG], (-)-cannabigerovarin [CBGV] and (-)-Δ^9-tetrahydrocannabivarin [THCV]).

Viability and proliferation of human SZ95 sebocytes were investigated by MTT- and CyQUANT-assays; cell death and lipid synthesis were monitored by $DiIC_1(5)$-SYTOX Green labelling and Nile Red staining, respectively. Inflammatory responses were investigated by monitoring expressions of selected cytokines upon lipopolysaccharide treatment (RT-qPCR, ELISA).

Up to 10 μM, the phytocannabinoids only negligibly altered viability of the sebocytes, whereas high doses (≥50 μM) induced apoptosis. Interestingly, basal sebaceous lipid synthesis was differentially modulated by the substances: CBC and THCV suppressed it, CBDV had only minor effects, whereas CBG and CBGV increased it. Importantly, CBC, CBDV and THCV significantly reduced arachidonic acid (AA)-induced "acne-like" lipogenesis. Moreover, THCV suppressed proliferation, and all phytocannabinoids exerted remarkable anti-inflammatory actions.

Our data suggest that CBG and CBGV may have potential in the treatment of dry-skin syndrome, whereas CBC, CBDV and especially THCV show promise to become highly efficient, novel anti-acne agents. Moreover, based on their remarkable anti-

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inflammatory actions, phytocannabinoids could be efficient, yet safe novel tools in the management of cutaneous inflammations.

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Key words: phytocannabinoid, acne vulgaris, cutaneous inflammation, dry skin

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Introduction

Acne is one of the most common human skin diseases (1). Although it is not a directly life-threatening one, it is well-documented that – especially its severe forms – can profoundly impair quality of life of the patients and, through social stigmatization, can lead to secondary psychological disorders (2,3). Despite extensive research efforts of the past decades, delicate details of the pathogenesis are still not completely unveiled. It is well-known that acne is a multifactorial disease; development and worsening of the symptoms can be triggered by psychological stress, alterations in the hormonal and nutritional status etc. (1,4-7). It is widely accepted that the first step of the pathogenesis is the increase in the sebum production, and alteration of its composition (1,5). Such alterations (e.g. desaturation of fatty acids, presence of lipoperoxides) as well as the decrease of the vitamin E content of the sebum were proven to trigger infundibular hyperkeratinisation leading to comedo formation. In the closed comedos bacteria (e.g. various *Propionibacterium acnes* strains) can overgrow, resulting in the acne-accompanying inflammatory processes of the sebaceous glands (SGs). Finally, the aforementioned alterations in the sebum composition also contribute to the worsening of the inflammation, which, in turn, further increases the sebum production, thereby closing the vicious circle of the pathogenesis (1,5,8).

Clinical treatment of acne is usually based on the severity of the symptoms and it is orchestrated in a step-wise, individualized manner (1,5). The most effective and versatile compound is 13-*cis*-retinoic acid (isotretinoin), which targets multiple steps of the pathogenesis at the same time (1,5). Although it is indeed highly efficient in

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alleviating the symptoms of acne, it usually induces severe side-effects (5,9-11). Hence, although we possess several drugs (e.g. topical or systemic isotretinoin, topical or systemic antibiotics, benzoyl peroxide, azelaic acid, anti-androgenic therapy; for detailed overview, see 1,5) acting through independent mechanisms, we still lack highly efficient yet safe therapeutic tools. Thus, there is an increasing need to explore the biology of the SGs and to identify novel therapeutic approaches with "favourable" side-effect profile.

Unfortunately, the regrettable lack of reliable animal models being capable to mimic the whole complexity of acne (1,7,12-15) make such investigations quite difficult. Therefore, we used a widely accepted *in vitro* model system, i.e. the human immortalized SZ95 sebocyte cell line (7,12-15) to study the role of one, selected aspect of the complex cutaneous neuroendocrine system (16-17) in the biology of the SGs, i.e. the so-called endocannabinoid system (18,19,s1-3; further details about it can be found in the **Supplementary Introduction section 1**). We showed that the prototypic endocannabinoids (i.e. anandamide [AEA] and 2-arachidonoylglycerol [2-AG]) are produced in human SGs (20). Moreover, we also demonstrated that these locally synthesized auto- and paracrine mediators, acting through the cannabinoid $(CB)_2$ receptor → ERK1/2 MAPK → PPAR pathway, are important positive regulators of the sebaceous lipid synthesis (20). These results raised the possibility that elevation of the endocannabinoid tone of the SGs, by increasing the sebaceous lipid production, may play an important role in the initial step of the pathogenesis of acne, and thus, an "anti-cannabinoid" treatment could be beneficial against it. Interestingly, however, we found that a CB_1-antagonising non-psychotropic phytocannabinoid (pCB), (-)-cannabidiol (CBD; 21), exerted complex anti-acne effects (22). Indeed,

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without influencing either viability or basal sebaceous lipid synthesis, CBD normalized pro-acne agents (e.g. arachidonic acid [AA]) (1,23) induced seborrhoea-mimicking lipogenesis not only in a quantitative, but also in a qualitative manner. It also reduced proliferation of the sebocytes both *in vitro* and *ex vivo* in full-thickness human skin organ culture via the activation of transient receptor potential vanilloid (TRPV) 4 ion channels (24,25) and the subsequent inhibition of the pro-lipogenic ERK1/2 MAPK pathway (20) and down-regulation of nuclear receptor interacting protein 1 (NRIP1), a well-known positive regulator of the lipid storage in adipocytes (26). Moreover, CBD was also able to suppress Toll-like receptor (TLR)-2 and -4 activation-evoked pro-inflammatory responses of the sebocytes via the A_{2A} adenosine receptor → cAMP↑ → tribbles homolog 3 (TRIB3)↑ pathway, inhibiting thereby P65-NF-κB (22).

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Taking into consideration that CBD is already in use in the neurological clinical practice (in a 1:1 ratio with (-)-*trans*-Δ^9-tetrahydrocannabinol [THC], under the trade name Sativex® [GW Pharmaceuticals, Cambridge, UK]) (27) and CBD alone has entered an expanded access program in children with intractable epilepsies (Epidiolex®, GW Pharmaceuticals, Cambridge, UK), and the commonly accepted high tolerability of the non-psychotropic phytocannabinoids (pCBs) in general, we intended to identify further highly efficient, yet safe potential anti-acne pCBs. Therefore, within the confines of the current pioneering *in vitro* study, we investigated the effects of multiple non-psychotropic pCBs, namely (-)-cannabichromene (CBC), (-)-cannabidivarin (CBDV), (-)-cannabigerol (CBG), (-)-cannabigerovarin (CBGV) and (-)-Δ^9-tetrahydrocannabivarin (THCV) by using human SZ95 sebocytes (1,7,15) in order to select the most promising ones for future extensive *ex vivo* and *in vivo*

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experiments. Further details regarding the above pCBs can be found in the **Supplementary Introduction section 2**.

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Methods

Materials

Anandamide (AEA), 2-arachidonoylglycerol (2-AG) and arachidonic acid (AA) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA); γ-irradiated lipopolysaccharide from *Escherichia coli* 026:B6 (LPS) was obtained from Sigma-Aldrich (St. Louis, MO, USA). All pCBs, i.e. CBC, CBDV, CBG, CBGV and THCV were provided by GW Pharmaceuticals (Cambridge, UK). LPS was dissolved in filtered distilled water. The solvent of all other compounds was absolute ethanol (Sigma-Aldrich).

Cell culturing

Human immortalized SZ95 sebocytes, originated from human facial sebaceous glands (15), were cultured in Sebomed® Basal Medium (Biochrom, Berlin, Germany) supplemented with 10 (V/V)% foetal bovine serum (Life Technologies Hungary Ltd., Budapest, Hungary), 1 mM $CaCl_2$, 5 ng/ml human epidermal growth factor (Sigma-Aldrich), MycoZap™ Plus-CL (1:500; Lonza, Budapest, Hungary). The medium was changed every other day, and cells were sub-cultured at 60-70% confluence.

Determination of intracellular lipids

For quantitative measurement of sebaceous (neutral) lipid content, cells (20,000 cells/well) were cultured in 96-well "black-well/clear-bottom" plates (Greiner Bio-One, Frickenhausen, Germany) in quadruplicates, and were treated with compounds as indicated. Subsequently, supernatants were discarded, cells were washed twice with phosphate-buffered saline (PBS; 115 mM NaCl, 20 mM Na_2HPO_4, pH 7.4; all from

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Sigma-Aldrich), and 100 µl of a 1 µg/ml Nile Red (Sigma-Aldrich) solution in PBS was added to each well. The plates were then incubated at 37°C for 20 min, and fluorescence was measured on FlexStation™ II^{384} fluorometer or FlexStation 3 multi-mode microplate reader (Molecular Devices, San Francisco, CA, USA). Results are expressed as percentage of the relative fluorescence units in comparison with the vehicle controls using 485 nm excitation and 565 nm emission wavelengths.

Determination of cellular viability, apoptosis and necrosis

Detailed description of the determination of viability, apoptosis and necrosis can be found in the **Supplementary Methods** section. Briefly, the viability of the cells was determined by measuring the conversion of the tetrazolium salt MTT (Sigma-Aldrich) to formazan by mitochondrial dehydrogenases. Apoptosis was determined by monitoring the decrease in the mitochondrial membrane potential, which is one of the earliest markers of apoptosis (28,29) using a MitoProbe™ $DilC_1(5)$ Assay Kit (Life Technologies Hungary Ltd.). Necrotic processes were determined by SYTOX Green staining (Life Technologies Hungary Ltd.). The dye is able to penetrate (and then bind to the nucleic acids) only to necrotic cells with ruptured plasma membranes, whereas healthy cells with intact surface membranes show negligible SYTOX Green staining. As a positive control for apoptosis and necrosis carbonyl cyanide m-chlorophenyl hydrazone (CCCP; Life Technologies Hungary Ltd.) and lysis buffer (Life Technologies Hungary Ltd.) were applied, respectively. An example for typical apoptotic and necrotic alterations are shown in **Fig. S1**.

Determination of cellular proliferation

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The degree of cellular growth (reflecting proliferation) was determined by measuring the DNA content of cells using CyQUANT Cell Proliferation Assay Kit (Life Technologies Hungary Ltd.). SZ95 sebocytes (2,000 cells per well) were cultured in "black-well/clear-bottom" 96-well plates (Greiner Bio-One) and were treated as indicated for 24, 48 and 72 hrs. Supernatants were then removed by blotting on paper towels, and the plates were subsequently frozen at -80°C. The plates were then thawed at room temperature, and 200 µl of CyQUANT dye/cell lysis buffer mixture was added to each well. After 5 minutes of incubation, fluorescence was measured at 490 nm excitation and 520 nm emission wavelengths using FlexStation 3 multi-mode microplate reader (Molecular Devices). Relative fluorescence values were expressed as percentage of 24-hr vehicle control regarded as 100%.

RNA isolation, reverse transcription and quantitative "real-time" PCR (Q-PCR)

Q-PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) or Stratagene Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA) using the 5' nuclease assay. Total RNA was isolated using TRIzol (Life Technologies Hungary Ltd.), DNase treatment was performed according to the manufacturer's protocol, and then 1 µg of total RNA was reverse-transcribed into cDNA by using High Capacity cDNA Kit from Life Technologies Hungary Ltd. PCR amplification was performed by using the TaqMan primers and probes (assay ID-s: Hs00174092_m1 for *interleukin* [*IL*]-*1α*, Hs00174097_m1 for *IL-1β*, Hs00985639_m1 for *IL-6*, Hs00174103_m1 for *IL-8*, Hs00174128_m1 for *tumour necrosis factor* [*TNF*]-*α*) and the TaqMan universal PCR master mix protocol (Applied Biosystems). As internal control, transcripts of *18S RNA* were determined (assay ID: Hs99999901_s1). The amount of the transcripts was

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normalized to those of the housekeeping gene using the ΔCT method. Finally, the results were normalized to the expression of the vehicle control (ΔΔCT method).

Determination of cytokine release (ELISA)

Cells were treated as indicated for 3 hours. Supernatants were collected, and the released amount of IL-6, and IL-8 was determined using OptEIA kits (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer's protocol.

Statistical analysis

Data were analysed by Origin Pro Plus 6.0 software (Microcal, Northampton, MA, USA), using Student's two tailed, two samples *t*-test (paired comparisons) or one-way ANOVA with Bonferroni's *post hoc* test (multiple comparisons) and $P<0.05$ values were regarded as significant differences. Graphs were plotted by using Origin Pro Plus 6.0 software (Microcal).

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Results

pCBs influence viability of sebocytes in a dose-dependent manner

First, we assessed the effects of the selected pCBs on the viability of SZ95 sebocytes using the MTT-assay. We found that, up to 10 μM, the investigated substances did not substantially decrease the viability of sebocytes upon 24- and 48-hr treatments (**Fig. S2a-b**). Although 100 nM CBDV and 500 nM CBG significantly decreased cell viability (24-hr treatments), these alterations were not present after 48-hr treatments, therefore they are most probably biologically irrelevant (**Fig. S2a-b**). On the other hand, we found that high concentrations of the pCBs (i.e. ≥50 μM) significantly reduced viability in all cases even after short-term (i.e. 24-hr) treatments (**Fig. S3**). These findings highlight the possibility of the onset of early apoptotic and necrotic processes, resulting in no obvious alterations in the MTT-assay upon treatment with lower (i.e. ≤10 μM) pCB concentrations. Therefore, in order to investigate such putative early effects, a combined fluorescent labelling was performed to simultaneously monitor mitochondrial membrane potential (decrease of which is a hallmark of early apoptotic processes; (28,29); $DilC_1(5)$ labelling) and necrosis-accompanying membrane disintegration (SYTOX Green staining). Importantly, we found that (except for some minor alterations seen in the case of CBC and THCV), up to 10 μM, none of the pCBs exerted biologically relevant pro-apoptotic or necrotic effects following their 24-hr treatments (**Fig. S4a-e**; note that an example of typical apoptotic and necrotic processes showing characteristic $DilC_1(5)$ and SYTOX Green alterations can be seen in **Fig. S1**). On the other hand, we also demonstrated that administration of high (≥50 μM) concentrations led to chiefly apoptotic cell death in all cases (24-hr treatments; **Fig. S5**).

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pCBs differentially influence basal sebaceous lipogenesis

Next, we aimed to study the effects of the pCBs on the basal sebaceous lipid production of the sebocytes, by using fluorescent Nile Red staining. As expected, when applied at cytotoxic (i.e. ≥50 μM) concentrations, all compounds suppressed basal sebaceous lipid synthesis during the course of the 24-hr treatments (**Fig. S6**). Of great importance, however, at non-cytotoxic doses (i.e. ≤10 μM; 48-hr treatments), the tested pCBs exerted markedly different actions on the lipogenesis, allowing us to categorize the substances into three major groups: “endocannabinoid-like”, i.e. pro-lipogenic (CBG and CBGV), “neutral” (CBDV) and “lipostatic” (CBC and THCV) ones (**Fig. 1a-e**). Indeed, quite surprisingly, CBG and CBGV induced a small (cca. 20-30% as compared to the control), yet significant increase in the sebaceous lipid synthesis (**Fig. 1a-b**). In contrast to CBG and CBGV, CBDV behaved in a more “CBD-like” way (22) having only negligible effects on the basal sebaceous lipid production (**Fig. 1c**), whereas CBC and THCV substantially suppressed it (**Fig. 1d-e**). Collectively, these findings suggest that CBC, CBDV and THCV, unlike CBG and CBGV, might have promising “CBD-like” anti-acne potential.

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CBG and CBGV abrogate AEA-induced lipogenesis

To further dissect the quite surprising pro-lipogenic actions of CBG and CBGV, and to investigate if there are any synergistic effects between the endo- and pCBs, next we co-administered them with AEA. Intriguingly, we found that CBG (10-20 μM) and CBGV (20 μM) were able to significantly suppress AEA-induced lipogenesis (24-hr treatments; **Fig. S7a-b**), raising the possibility that CBG as well as CBGV might act

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as partial (i.e. less efficacious) agonists of the same pro-lipogenic signalling pathway as AEA.

THCV, CBC and CBDV suppress AA-induced seborrhoea-mimicking lipogenesis

As it has already been mentioned above, the key initial step of the acne pathogenesis is the elevation and characteristic qualitative alteration of the sebum production. Therefore, normalization of this is one of the key goals of the acne therapy (1,5). Thus, next, we tested their efficiency in normalizing AA-induced, “acne-like”, seborrhoea-mimicking lipid synthesis (1,22,23). As revealed by Nile Red staining, both CBC and CBDV were able to dose-dependently reduce AA-induced neutral lipid synthesis (**Fig. 2a-b**). However, their efficiency was far exceeded by THCV, which, similar to CBD (22), was able to completely normalize AA-induced sebaceous lipid synthesis (**Fig. 2c**). In order to investigate whether this remarkable lipostatic action of THCV was specific to the AA-induced, protein kinase C (PKC)-δ-mediated (23) lipogenesis, or it developed due to the activation of a universal lipostatic pathway, we also studied its efficiency against two prototypic endocannabinoids, i.e. the aforementioned AEA and 2-AG (20). We found that, again, similar to CBD (22), 10 μM of THCV was able to fully abrogate lipogenic actions of both endocannabinoids (48-hr treatments; **Fig. S8a-b**) indicating that it indeed normalizes lipogenesis in a “CBD-like”, universal manner.

THCV suppresses sebocyte proliferation

As sebum production is realized via holocrine secretion, *in vivo* it is greatly dependent not only on the sebaceous lipid production of the individual sebocytes, but also on the number of the cells in the sebaceous glands (1,5,7,30,31). Thus,

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suppressing proliferation of the sebocytes (ideally without compromising their viability) is one of the key requirements of a proper anti-acne agent. Since our aforementioned results on lipogenesis suggested that among the tested compounds THCV might have the greatest potential to be an efficient, novel anti-acne agent, we also assessed its effects on the proliferation of sebocytes by using CyQUANT-assay. We found that non-cytotoxic concentrations (≤10 μM) of THCV exerted a dose-dependent anti-proliferative action in course of 72-hr treatments. Moreover, at its highest test concentration it appeared to stop the proliferation completely (**Figure 3**), demonstrating remarkable sebostatic (i.e. lipostatic+anti-proliferative) activity *in vitro*. It is also noteworthy that, in agreement with our previous data (**Fig. S2a-b** and **S4e**), the cell count did not decrease below the level of the 24-hr control (**Fig. 3**), indicating a "pure" anti-proliferative action without any cytotoxic activity.

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THCV exerts strong anti-inflammatory actions

Besides the pathologically elevated lipogenesis and proliferation, another "sebocyte-specific" acne modality is inflammation (1,5,8). Thus, based on the above data, we decided to investigate the effects of THCV on the immune properties of the sebocytes. Pro-inflammatory response was induced via activating Toll-like receptor (TLR)-4 by using bacterial lipopolysaccharide (LPS). As expected based on our previous results (22), LPS-treatment resulted in a characteristic pro-inflammatory response as revealed by Q-PCR and ELISA (3-hr treatments; **Fig. 4a-c**). Of great importance, THCV was able to fully abolish LPS-induced elevations in expressions of several well-known, "acne-relevant" pro-inflammatory cytokines (1,32), i.e. *IL-1α*, *IL-1β*, *IL-6*, *IL-8* and *TNF-α* (**Fig. 4a**). In order to further confirm the anti-inflammatory action of THCV, we measured the concentrations of the released IL-6 and IL-8 in the

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supernatant of the sebocytes. Of great importance, we found that release of IL-6 and IL-8 was efficiently inhibited by co-administration of THCV (**Fig. 4b-c**), indicating that this pCB indeed exerted complex anti-acne effects targeting all the three key "sebocyte-specific" steps of the acne pathogenesis.

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Discussion & Conclusions

Within the confines of the current pioneering *in vitro* study, we investigated the effects of selected non-psychotropic pCBs, and here we present the first evidence that not only CBD, but also additional plant-derived cannabinoids (i.e. CBC, CBDV and especially THCV) exert potent anti-acne effects *in vitro*. According to our results, although CBC and CBDV also had significant lipostatic activity, THCV was the most promising anti-acne compound. Indeed, we found that, similar to CBD (22), it exerted universal lipostatic actions (**Fig. 2c**; **S8a-b**). It was able to fully abrogate "acne-mimicking" activity of substances acting through independent pro-lipogenic signalling pathways (i.e. the effects of the PKCδ-activating AA (23) as well as of AEA and 2-AG, primarily targeting the CB_2R) (20). Moreover, without compromising the viability, THCV suppressed proliferation of the sebocytes (**Fig. 3**), which, due to the holocrine mechanism of the sebum production (1), could be a very important finding from the point-of-view of the putative *in vivo* efficiency. Last, but not least, THCV successfully abrogated LPS-induced pro-inflammatory responses as well (**Figure 4a-c**), influencing thereby all the sebocyte-specific aspects of acne to a highly desirable direction. These findings make THCV a very promising candidate drug in the management of acne. Hence, clinical studies are urgently invited to explore its putative *in vivo* efficiency. Further discussion of the putative additional anti-acne effects of future cannabinoid-based medications, with a special emphasis on THCV can be found in the **Supplementary Discussion & Conclusions section 1**.

It should also be noted that, according to our preliminary findings, not only THCV, but also CBG, CBGV, CBC and CBDV suppressed LPS-induced pro-inflammatory

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response of the sebocytes (**Fig. S9a-e**). These findings together with the known anti-proliferative actions of the pCBs (**Fig. 3**) (33) raise the possibility that administration of these substances may be beneficial not only in acne, but also in other inflammation-accompanied skin diseases, e.g. in psoriasis, a disease characterized by cutaneous inflammation, as well as hyperproliferation and disturbed differentiation of the keratinocytes (34,35).

Our data introduced THCV as the most potent anti-acne agent among the five investigated pCBs, being highly likely to exert its sebostatic actions in a “CBD-like” way, i.e. via the activation of certain TRPV channels. Considering that THCV exerted the most effective lipostatic action (almost complete normalization of the AA-induced lipogenesis in contrast to the partial efficiency of CBC and CBDV; **Fig. 2a-c**), one could speculate that this pCB might be more efficacious in activating such TRPV channels as compared to CBC, CBDV, CBG or CBGV. Of great importance, this hypothesis is perfectly in line with the data of the literature. Indeed, by using rat TRPV3 and -4 overexpressing HEK-293 cells, De Petrocellis *et al.* (2012) demonstrated that among the five tested substances, THCV possessed the highest efficacy in activating TRPV3 and -4 (36). Moreover, THCV was found to activate these channels in the low micromolar range (EC_{50} values for TRPV3 and TRPV4 were found to be 3.8±0.4 and 6.4±0.7 μM, respectively) (36), i.e. in the exact same range in which its strongest lipostatic and anti-proliferative actions occurred (**Fig. 1e, 2c, 3; S8a-b**). Moreover, THCV was also found to be potent activator of human TRPV1 and rat TRPV2 (37), which means that it was proven to be capable of activating all four TRPV channels (namely TRPV1, -2, -3 and -4), which are known to negatively regulate sebaceous lipid synthesis of the sebocytes (22,38,39). Although

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affinity of THCV to human TRPV channels might differ from its affinity to rat TRPV channels, our results suggest that beneficial sebostatic actions of THCV might be mediated via activating some (if not all) of the above TRPV channels.

Surprisingly, our results also revealed a very intriguing functional heterogeneity between the pCBs: CBG and CBGV, in contrast to CBC, CBDV and THCV, behaved in an "endocannabinoid-like" way, and increased sebaceous lipid synthesis of the sebocytes (**Fig. 1a-b**) raising the possibility of their administration in the management of conditions, such as dry skin syndrome, xerosis and even skin ageing (40-43). Thus, although it was not our primary goal, our findings shed some light on the complexity of the signalling pathways activated by the different pCBs: our results on the lipid synthesis (**Fig. 1**) indicate that the five pCBs may exert their effects by activating a markedly different target molecule pattern and/or their efficacies in activating these targets may be different. Unfortunately, apart from THC, CBD and CBG, little is known about the cellular targets of the remaining pCBs. It was already shown that CBG is an agonist of α2 adrenoceptor, TRPA1, TRPV1 and TRPV2; it activates but rapidly desensitizes TRPV3 and TRPV4; it antagonizes CB_1, TRPM8 and 5-HT_{1A} receptors; and it inhibits the endocannabinoid membrane transporter (36,44,45). Interestingly, although CBG is known to bind to CB_2, it is not clear whether it exerts agonistic or antagonistic effects on this receptor (44). Importantly, recent findings of Carrillo-Salinas *et al.*, demonstrated that a CBG-quinone compound ("VCE-003") activated CB_2 and PPARγ (46), i.e. receptors having already been shown to be important players in mediating lipogenic actions of the endocannabinoids (20). These data together with our findings presented here; i.e. that CBG as well as CBGV (i) increased basal sebaceous lipogenesis (**Fig. 1a-b**); (ii)

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3 this elevation was below the ones usually exerted by the endocannabinoids (~20-
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5 30% vs. ~100%; **Fig. 1a-b**; **S8a-b**); and (iii) they reduced AEA-induced lipid synthesis
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7 (**Fig. S7a-b**), indicated that CBG (and perhaps also CBGV) might indeed be partial
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10 CB_2 (and/or PPARγ) agonists at ~10-20 μM.
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14 It should also be noted that pCBs are usually well-tolerated drugs even in the case of
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16 oral administration (27,47,48), and some of them are already in use in the clinical
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19 practice, e.g. dronabinol (international non-proprietary name of (-)-*trans*-Δ^9-
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21 tetrahydrocannabinol [THC]) in Marinol® (AbbVie Inc. North Chicago, Illinois, USA)
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23 (49), nabilone (a synthetic "THC-like" cannabinoid) in Cesamet (Valeant
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25 Pharmaceuticals International Inc., Montreal, Canada) (49), or THC and CBD in
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27 Sativex® (GW Pharmaceuticals, Cambridge, UK) (27). Moreover, they all are highly
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29 lipophilic compounds, therefore in case of topical application, they are likely to
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31 penetrate to the skin via the transfollicular route and thereby accumulate in the lipid-
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34 rich sebaceous glands (50). Thus, physicochemical properties of the pCBs make
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36 them ideal drugs to target selectively the SGs by using topical formulations, which
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38 could also minimize the risk of the occurrence of systemic side-effects (further
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40 discussion of the putative systemic and local adverse effects can be found in the
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42
43 **Supplementary Discussion & Conclusions section 2**).
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47 Obviously, despite of the promising results, a significant limitation of the current study
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49 is that, due to the aforementioned lack of reliable animal models (1,7,12-15), it was
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52 based on a single cell line. Clinical studies are therefore urgently invited to exploit
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54 putative therapeutic potential of CBG, CBGV, CBC, CBDV, THCV and maybe other
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non-psychotropic pCBs in the management of various inflammation-accompanied cutaneous diseases, and especially the one of THCV in alleviating acne.

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Conflict of interest

CC Zouboulis owns an international patent on the SZ95 sebaceous gland cell line (WO2000046353). C Stott is employed by GW Pharmaceuticals.

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Figure legends

Figure 1 *Non-psychotropic pCBs differentially influence basal sebaceous lipid synthesis of human sebocytes*

(**a-e**) Sebaceous lipid production of SZ95 sebocytes was assessed by Nile Red staining following 48-hr treatments. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of four independent determinations. Two additional experiments yielded similar results. *, ** and *** mark significant (P<0.05, 0.01 or 0.001, respectively) differences compared to the vehicle control. CBC: (-)-cannabichromene; CBDV: (-)-cannabidivarin; CBG: (-)-cannabigerol; CBGV: (-)-cannabigerovarin; THCV: (-)-Δ^9-tetrahydrocannabivarin.

Figure 2 *THCV, CBC and CBDV exert remarkable lipostatic actions in AA-induced model seborrhoea*

(**a-c**) Sebaceous lipid production of SZ95 sebocytes was measured by Nile Red staining following 48-hr treatments. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of four independent determinations. Two additional experiments yielded similar results. ** and *** mark significant (P<0.01 or 0.001, respectively) differences compared to the vehicle control. AA: arachidonic acid; CBC: (-)-cannabichromene; CBDV: (-)-cannabidivarin; THCV: (-)-Δ^9-tetrahydrocannabivarin.

Figure 3 *Non-cytotoxic concentrations of THCV exert strong anti-proliferative effect on human sebocytes*

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CyQUANT proliferation assay after 24-, 48- and 72-hr treatments. Results are expressed in the percentage of the 24-hr vehicle control (100%, solid line) as mean±SEM of four independent determinations. One additional experiment yielded similar results. *** marks significant (P<0.001) differences compared to the 24-hr vehicle control, whereas ## and ### indicate significant (P<0.01 or 0.001, respectively) differences compared to the vehicle control of the same day. THCV: (-)-Δ^9-tetrahydrocannabivarin.

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Figure 4 *THCV exerts remarkable anti-inflammatory actions*

(**a**) Q-PCR analyses of SZ95 sebocytes following the indicated 3-hr simultaneous treatments. Data are presented by using the ΔΔCT method regarding 18S RNA-normalized mRNA expressions of the vehicle control as 1 (solid line). Data are expressed as mean±SD of 2-3 determinations. One additional experiment yielded similar results. (**b-c**) Determination of the released cytokine concentration following 3-hr simultaneous treatments. Data are presented as mean±SEM of three determinations. One additional experiment yielded similar results. (**a-c**) *** marks significant (P<0.001) differences as indicated. THCV: (-)-Δ^9-tetrahydrocannabivarin.

Figure 1 *Non-psychotropic pCBs differentially influence basal sebaceous lipid synthesis of human sebocytes*

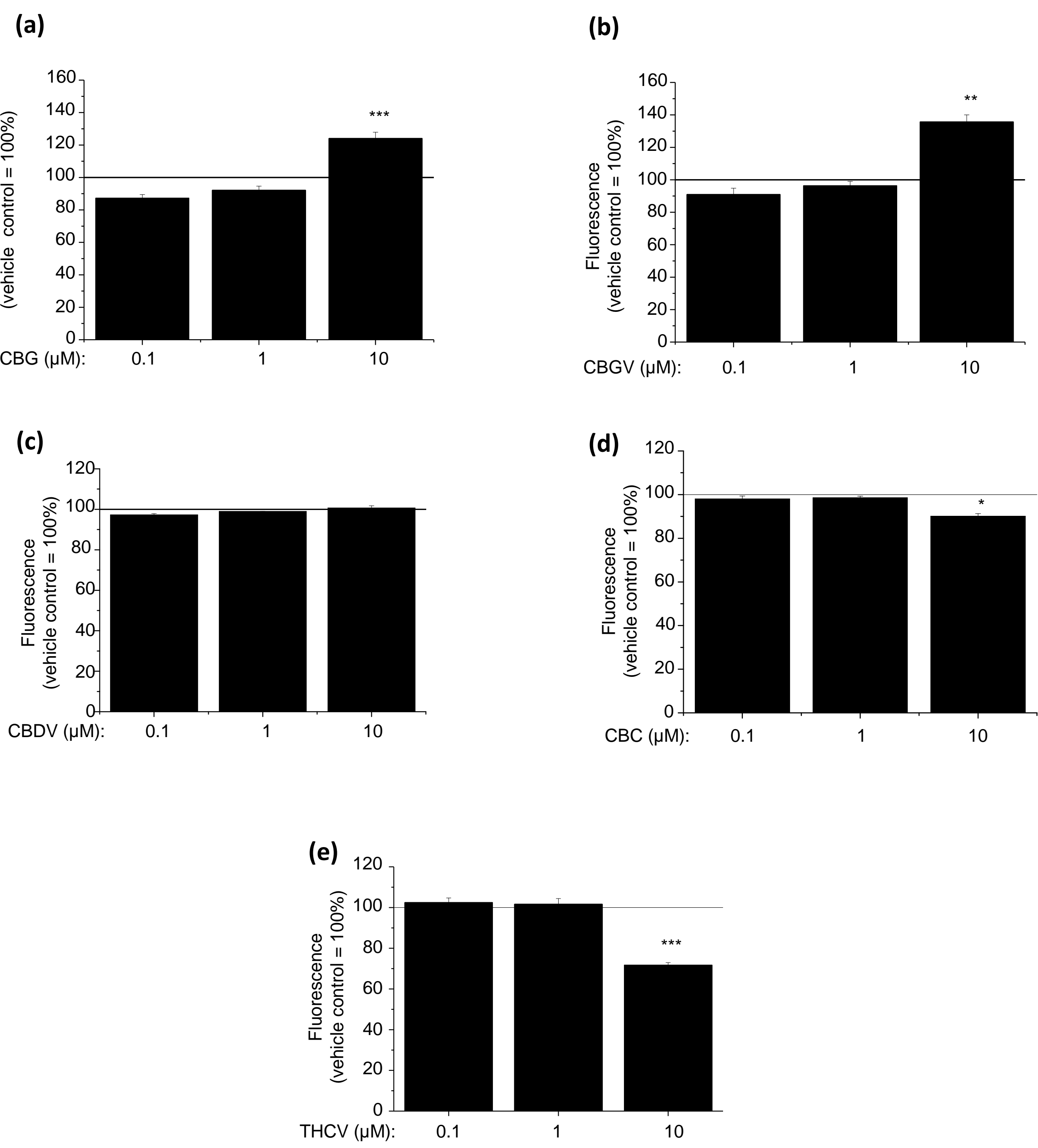

Figure 2 *THCV, CBC and CBDV exert remarkable lipostatic actions in AA-induced model seborrhoea*

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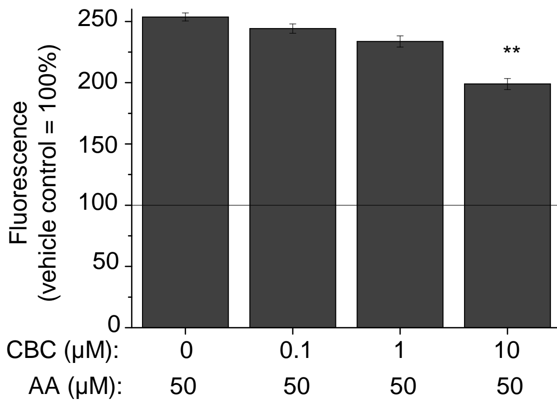
(a)



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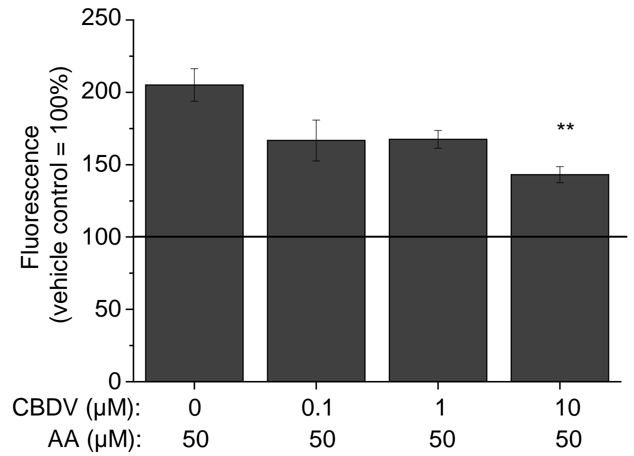

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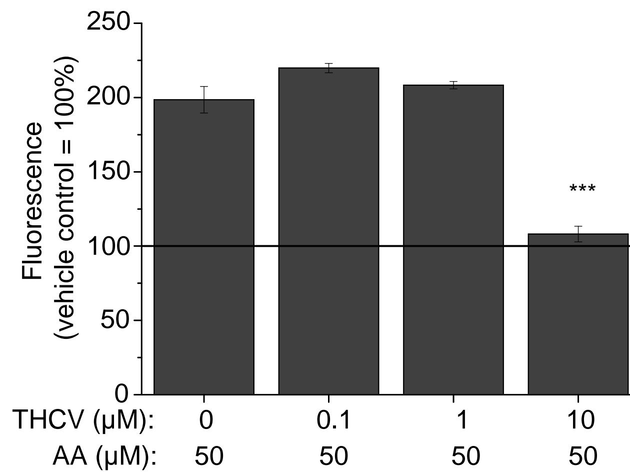

Figure 3 *Non-cytotoxic concentrations of THCV exert strong anti-proliferative effect on human sebocytes*

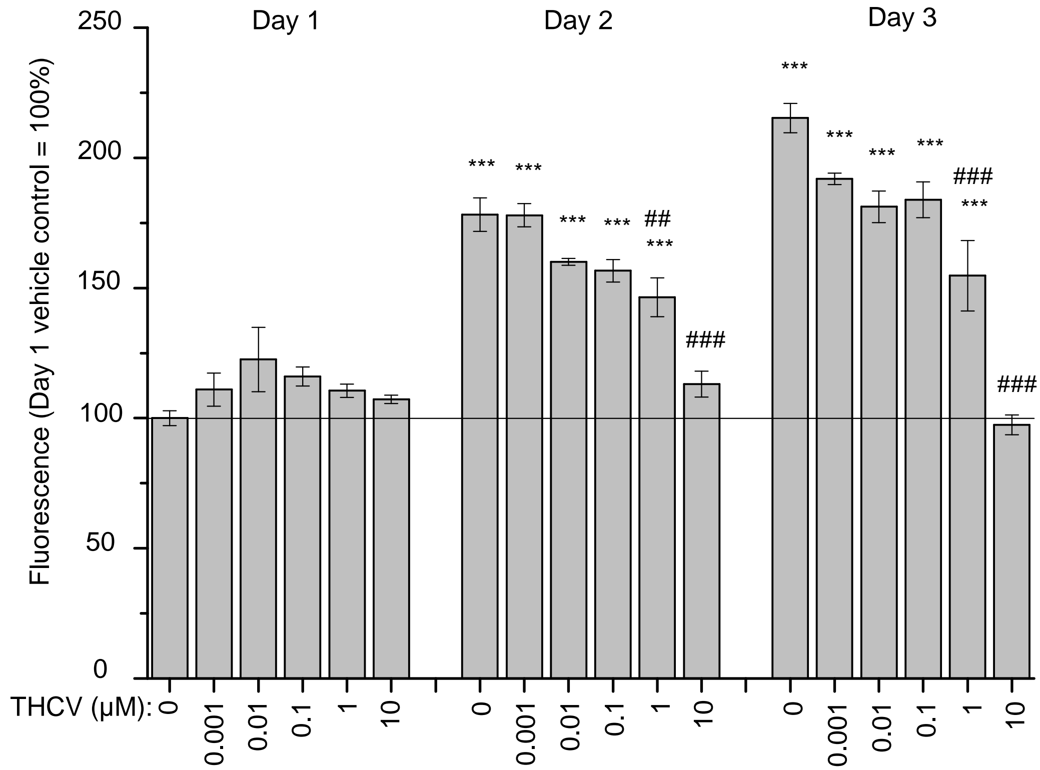

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Figure 4 *THCV exerts remarkable anti-inflammatory actions*

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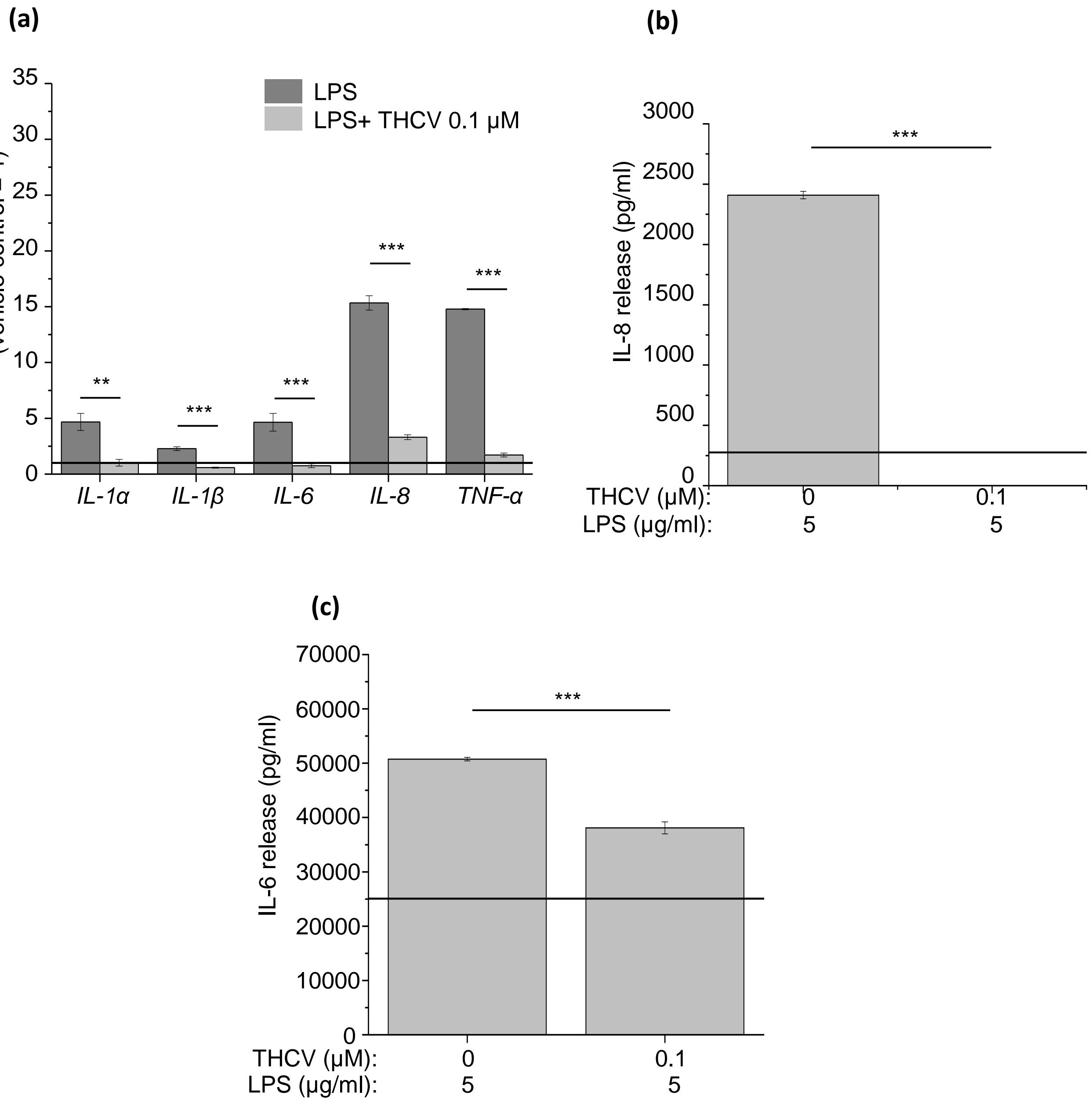

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SUPPLEMENTARY MATERIAL

Differential effectiveness of selected non-psychotropic phytocannabinoids on human sebocyte functions implicates their introduction in dry/seborrheic skin and acne treatment

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Supplementary Introduction section 1

The endocannabinoid system

The endocannabinoid system (ECS) is a complex signalling network comprising endogenous ligands (i.e. the "endocannabinoids" [eCB], such as arachidonoylethanolamine, also known as anandamide [AEA] or 2-arachidonoylglycerol [2-AG], etc.), various cannabinoid-sensitive receptors (e.g. cannabinoid [CB]-1 and -2, several transient receptor potential [TRP] channels, etc.) as well as enzymes and transporters being responsible for the synthesis (e.g. N-acyl phosphatidylethanolamine specific phospholipase D, diacylglycerol lipases-α and –β) and degradation (fatty acid amide hydrolase [FAAH], monoacylglycerol lipase, endocannabinoid membrane transporter [EMT]) of the eCBs (18,19,s1-3).

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The ECS was shown to regulate various physiological functions all over the body. In the central nervous system (CNS), it regulates behaviour, mood, appetite, and memory in a chiefly CB_1-dependent manner, whereas its best-characterized activities at the periphery are coupled to the regulation of the immune system via the activation of CB_2 (18,19,s1-3). Therefore, although both receptors can be found in both locations, CB_2 is very often referred to be the "anti-inflammatory, peripheral", whereas CB_1 the "psychotropic, central" cannabinoid receptor.

Besides, ECS has also been proven to be functionally active in the (human) skin. Indeed, in a wider context, cutaneous ECS is an important part of the complex neuroendocrine system of the skin (extensively reviewed by Slominski et al.; 16,17), controlling several fundamental cutaneous functions, such as e.g. hair growth, sebaceous lipid production, mast cell maturation and degranulation, fibroblast

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functions, melanogenesis, keratinocyte proliferation and differentiation, etc. (for details, see 19,20,s4-5).

Supplementary Introduction section 2

Plant-derived or "phyto"-cannabinoids (pCBs) are characteristic molecules of the plant *Cannabis sativa*. Among the cca. 100 different pCBs (-)-*trans*-Δ^9-tetrahydrocannabinol (THC) is the best studied one, since it is the major responsible for the CB_1 receptor-coupled psychotropic effects of the *Cannabis*-derived drugs, i.e. marijuana and hashish (19,20,s2). Of great importance, the vast majority of the remaining pCBs are non-psychotropic ones due to the lack of CB1 agonism (21,s6-10). Since one of the greatest challenges of the cannabinoid-based drug development is to avoid CB_1-coupled psychotropic side-effects (19,20,s1-2), these pCBs are exceptional candidates for future drug development.

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Supplementary Methods

Determination of cellular viability

The viability of the cells was determined by measuring the conversion of the tetrazolium salt MTT (Sigma-Aldrich) to formazan by mitochondrial dehydrogenases. Cells were plated in 96-well plates (20,000 cells/well) in quadruplicates, and were treated as indicated for 1 or 2 days. Cells were then incubated with 0.5 mg/ml MTT for 2 hrs, and concentration of formazan crystals (as an indicator of number of viable cells) was determined colorimetrically at 565 nm by using FlexStation 3 multi-mode microplate reader (Molecular Devices). Results were expressed as percentage of vehicle controls regarded as 100%.

Determination of apoptosis

A decrease in the mitochondrial membrane potential is one of the earliest markers of apoptosis (28,29). Therefore, to assess the process, mitochondrial membrane potential of SZ95 sebocytes was determined using a MitoProbe™ $DiIC_1(5)$ Assay Kit (Life Technologies Hungary Ltd.). Cells (20,000 cells/well) were cultured in 96-well “black-well/clear-bottom” plates (Greiner Bio One) in quadruplicates and were treated as indicated for 24 hrs. After removal of supernatants, cells were incubated for 30 minutes with $DiIC_1(5)$ working solution (50 µl/well), then washed with PBS, and the fluorescence of $DiIC_1(5)$ was measured at 630 nm excitation and 670 nm emission wavelengths using FlexStation™ II^{384} fluorometer or FlexStation 3 multi-mode microplate reader (Molecular Devices). Relative fluorescence values were expressed as percentage of vehicle controls regarded as 100%. As a positive control for apoptosis, we applied carbonyl cyanide m-chlorophenyl hydrazone (CCCP; Life

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Technologies Hungary Ltd.) dissolved in the DilC$_1$(5) working solution (1:200 for 30 min; **Fig. S1**).

Determination of necrosis

Necrotic processes were determined by SYTOX Green staining (Life Technologies Hungary Ltd.). The dye is able to penetrate (and then bind to the nucleic acids) only to necrotic cells with ruptured plasma membranes, whereas healthy cells with intact surface membranes show negligible SYTOX Green staining. Cells were cultured in 96-well “black-well/clear-bottom” plates (Greiner Bio One), and treated as indicated for up to 24 hrs. Supernatants were then discarded, and the cells were incubated for 30 minutes with 1 μM SYTOX Green dye. Following incubation, cells were washed with PBS, the culture medium was replaced, and fluorescence of SYTOX Green was measured at 490 nm excitation and 520 nm emission wavelengths using FlexStation™ II^{384} fluorometer or FlexStation 3 multi-mode microplate reader (Molecular Devices). Relative fluorescence values were expressed as percentage of vehicle controls regarded as 100%. As a positive control for necrosis, lysis buffer (1:100 in the SYTOX Green working solution for 30 min; Life Technologies Hungary Ltd.) was applied (**Fig. S1**).

Due to their spectral properties, DilC$_1$(5) and SYTOX Green dyes were always administered together, enabling us to investigate necrotic and early apoptotic processes of the same cultures. Selective decrease of DilC$_1$(5) intensity indicated mitochondrial depolarization (i.e. the onset of early apoptotic processes), whereas increase of SYTOX Green staining intensity revealed necrotic cell death. An example for typical apoptotic and necrotic alterations are shown in **Fig. S1**.

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Supplementary Discussion & Conclusions section 1

Acne is a multifactorial, inflammation-accompanied skin disease, detrimentally affecting quality of life of many patients world-wide (1,4-6). Although nowadays several therapeutic tools are available (1,5), we still lack highly efficient, yet safe medications; thus many studies intended to reveal such novel possibilities. We have previously shown that in human SGs the locally produced AEA and 2-AG promote sebaceous lipid synthesis via an auto- and paracrine regulation (20); one promising direction could therefore be the appropriate modulation of the ECS (i.e. by antagonizing CB_2 receptor or reducing the local endocannabinoid concentrations). On the other hand, our recently published data describing the potent and complex anti-acne effects of CBD *in vitro* and *ex vivo* (22) pointed to an entirely new direction, i.e. to the administration of certain non-psychotropic pCBs in the management of acne, and shed light on previously unknown sebostatic (TRPV4) and anti-inflammatory (A_{2A} adenosine receptors) therapeutic targets expressed by the sebocytes (22).

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THCV (similar to CBD) appears to be a multifaceted, promising, novel anti-acne agent efficiently targeting all the three key sebocyte-specific steps of the pathogenesis (i.e. increased sebaceous lipid synthesis, proliferation and inflammation). Furthermore, it should also be noted that pCBs in general are highly likely to have beneficial effects against two major additional, “sebocyte-independent” aspects of the acne pathogenesis, i.e. infundibular hyperkeratosis leading to comedogenesis and overgrowth of acnegenic bacteria (1,5). Indeed, several pCBs were already shown to inhibit differentiation (CBD and CBG) (s11), and to reduce proliferation of keratinocytes (e.g. CBD and CBG) (33) making them likely to be efficient in suppressing infundibular hyperkeratosis and subsequent comedogenesis,

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and many of them (e.g. CBD, CBC, CBG, etc.) were evidenced to exert potent anti-bacterial activity (s12), which augurs to be useful in countering colonization of acnegenic *Propionibacterium acnes* strains.

Supplementary Discussion & Conclusions section 2

As discussed above, pCBs are well-known to influence the activity of a wide-array of cellular targets, including receptors (CB_1, CB_2, 5-HT_{1A}, adenosine A_{2A}, GPR55, μ opioid) enzymes (FAAH, phospholipase A2, cyclooxygenase, lipoxygenase or certain cytochrome P450 enzymes, etc.), transporters (EMT, equilibrative nucleotide transporter 1, etc.), ion- and non-ion selective pores (certain TRP channels, 5-HT_3 receptor, voltage-dependent anion channel 1, etc.) (21,22,27,36,37,44-46,s3,s6-11,s13-22). Moreover, in some cases the development of indirect "*entourage*" effects should also be taken into consideration (s11,s14,s23).

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According to their varying affinities towards their targets, each pCB can be characterized by a more or less unique "fingerprint" of molecular actions, which determines the nature of its biological activity. The wide-array of possible interactions definitely raises the risk of unwanted and unexpected (!) adverse effects, therefore, although the pCBs being currently in use in the clinical practice possess a very "desirable" side-effect profile (for details, see **Discussion & Conclusions section** of the main text), introduction of novel (i.e. previously un-tested) pCB-based medications in the dermatological practice must be preceded by rigorous pre-clinical (e.g. mini-pig studies) and clinical studies to minimize the risk of any harmful actions (s24,s25).

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By administering pCBs, one shall take into consideration at least two major groups of side-effects, i.e. psychotropic actions due to the systemic distribution of the substances followed by the subsequent activation of the neuronal CB_1 expressed in the central nervous system, as well as local adverse effects. In our case the first option can almost certainly be ruled out due to the non-psychotropic nature of the investigated pCBs, which were already shown to lack CB_1 agonism in many systems (21,s6-10). Moreover, to further minimize the risk of any unexpected actions, specific topical formulations are to be developed to help the delivery of the pCBs into the skin, but (if possible) to prevent their transcutaneous absorption to the circulation.

On the other hand, we should not forget about the fact that, besides the sebocytes, pCBs may reach other cutaneous cells (epidermal keratinocytes, melanocytes, Langerhans cells, fibroblasts, cells of the hair follicles, etc.) as well. Although, as mentioned in the **Discussion & Conclusions section** of the main text, due to their physicochemical properties, topically administered pCBs are likely to target SGs with high selectivity (50) there is a small, but non-negligible chance to influence the biology of these cells and cutaneous structures as well. Obviously, these local additional (not necessarily adverse!) effects will greatly depend on the pharmacokinetic properties (pCB concentration, presence or absence of permeation enhancers, etc.) of the given formulations in which the pCB would be administered. Thus, upon development of such formulations, the aforementioned, routinely applied rigorous pre-clinical and clinical testing must be conducted in the case of the novel, pCB-based pharmaceuticals/cosmeceuticals.

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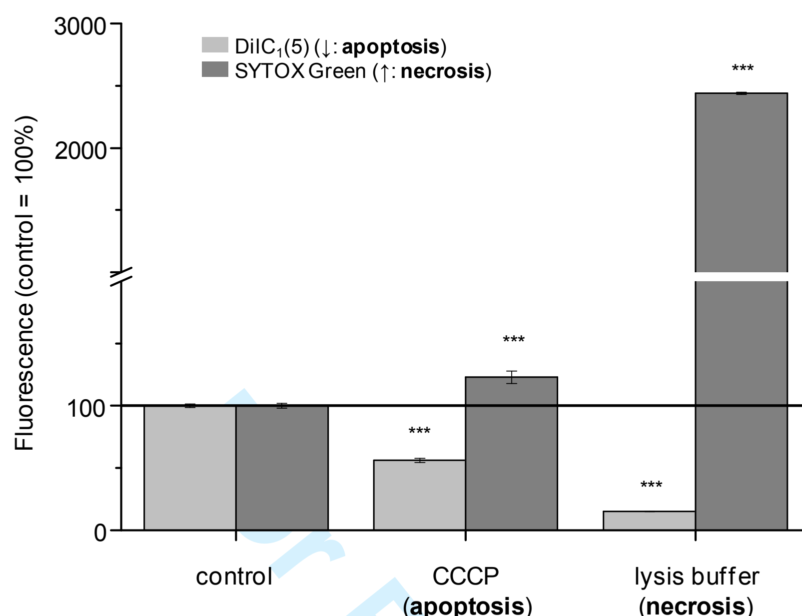
Supplementary Figures



Supplementary Figure S1 *Assessment of the effects of apoptosis and necrosis positive controls in human sebocytes*

Cell death of SZ95 sebocytes was investigated by combined fluorescent $DiIC_1(5)$-SYTOX Green labelling. As apoptosis positive control, carbonyl cyanide m-chlorophenyl hydrazone (CCCP; a well-known un-coupler of the mitochondrial inner membrane) was applied (1:200 for 30 min), whereas necrosis was induced by lysis buffer (1:100 for 30 min). *** marks significant (P<0.001) differences compared to the control group. Note that although CCCP (besides decreasing the mitochondrial membrane potential as revealed by $DiIC_1(5)$ signal intensity) significantly increased the SYTOX Green intensity, its action was far exceeded by lysis buffer, which induced a dramatic (>2,000%) increase in it. In general, selective >20% decrease of $DiIC_1(5)$ intensity (maybe accompanied by minor, i.e. <50% increase in the SYTOX Green staining) indicates the development of “pure” apoptotic processes, whereas simultaneous biologically relevant (i.e. >50%) increase of SYTOX Green level marks necrotic processes.

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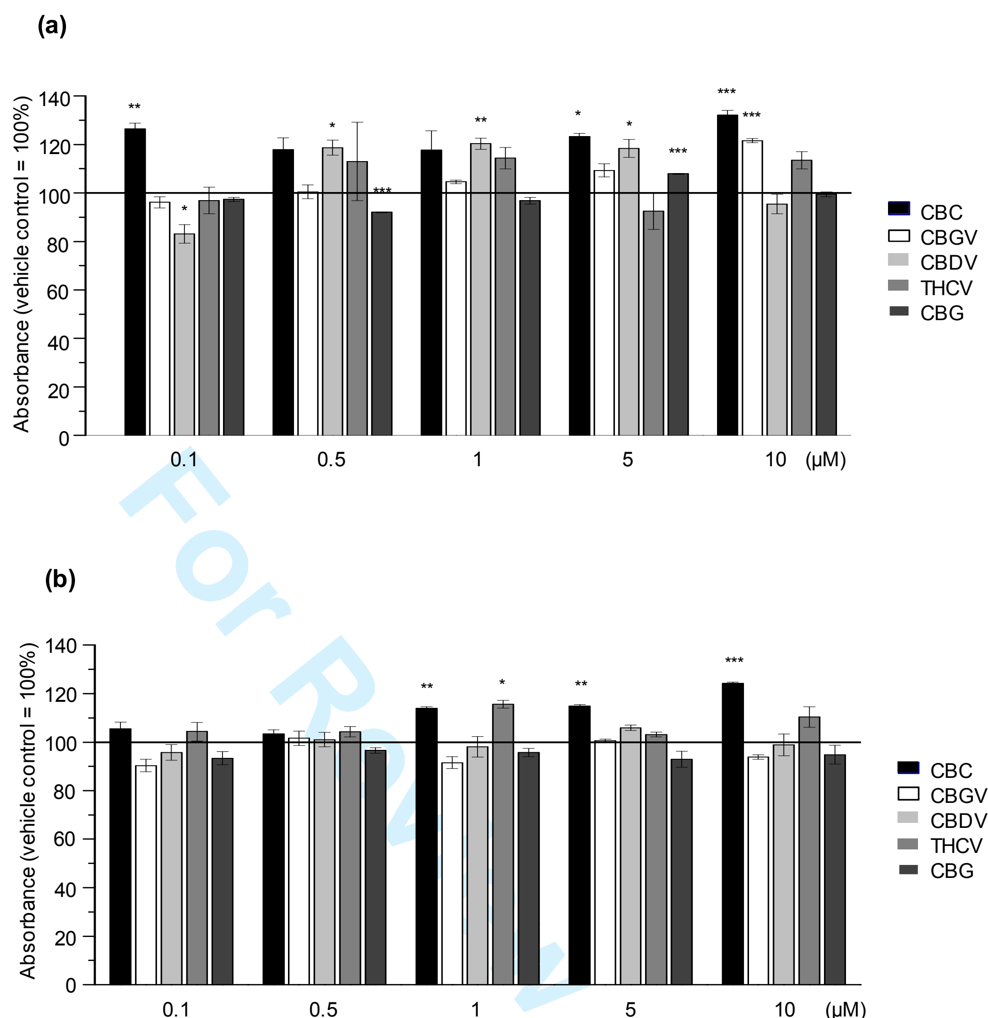

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Supplementary Figure S2 *Up to 10 µM, none of the investigated pCBs decreased viability of sebocytes following 24- (a) and 48-hr (b) treatments*

Viability of SZ95 sebocytes was monitored by MTT-assay following 24- (**a**) or 48-hr (**b**) treatments. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of four independent determinations. Two additional experiments yielded similar results. *, ** and *** mark significant (*P*<0.05, 0.01 or 0.001, respectively) differences compared to the vehicle control. CBC: (-)-cannabichromene; CBDV: (-)-cannabidivarin; CBG: (-)-cannabigerol; CBGV: (-)-cannabigerovarin; THCV: (-)-Δ^9-tetrahydrocannabivarin.

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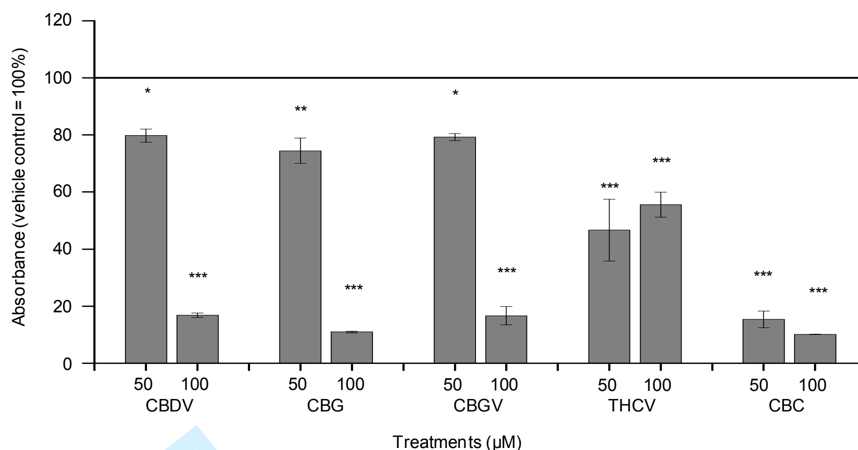

Supplementary Figure S3 *High concentrations of the pCBs decreased viability of sebocytes*

Viability of SZ95 sebocytes was monitored by MTT-assay following 24-hr treatments. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of four independent determinations. One additional experiment yielded similar results. *, ** and *** mark significant (P<0.05, 0.01 or 0.001, respectively) differences compared to the vehicle control. CBC: (-)-cannabichromene; CBDV: (-)-cannabidivarin; CBG: (-)-cannabigerol; CBGV: (-)-cannabigerovarin; THCV: (-)-Δ^9-tetrahydrocannabivarin.

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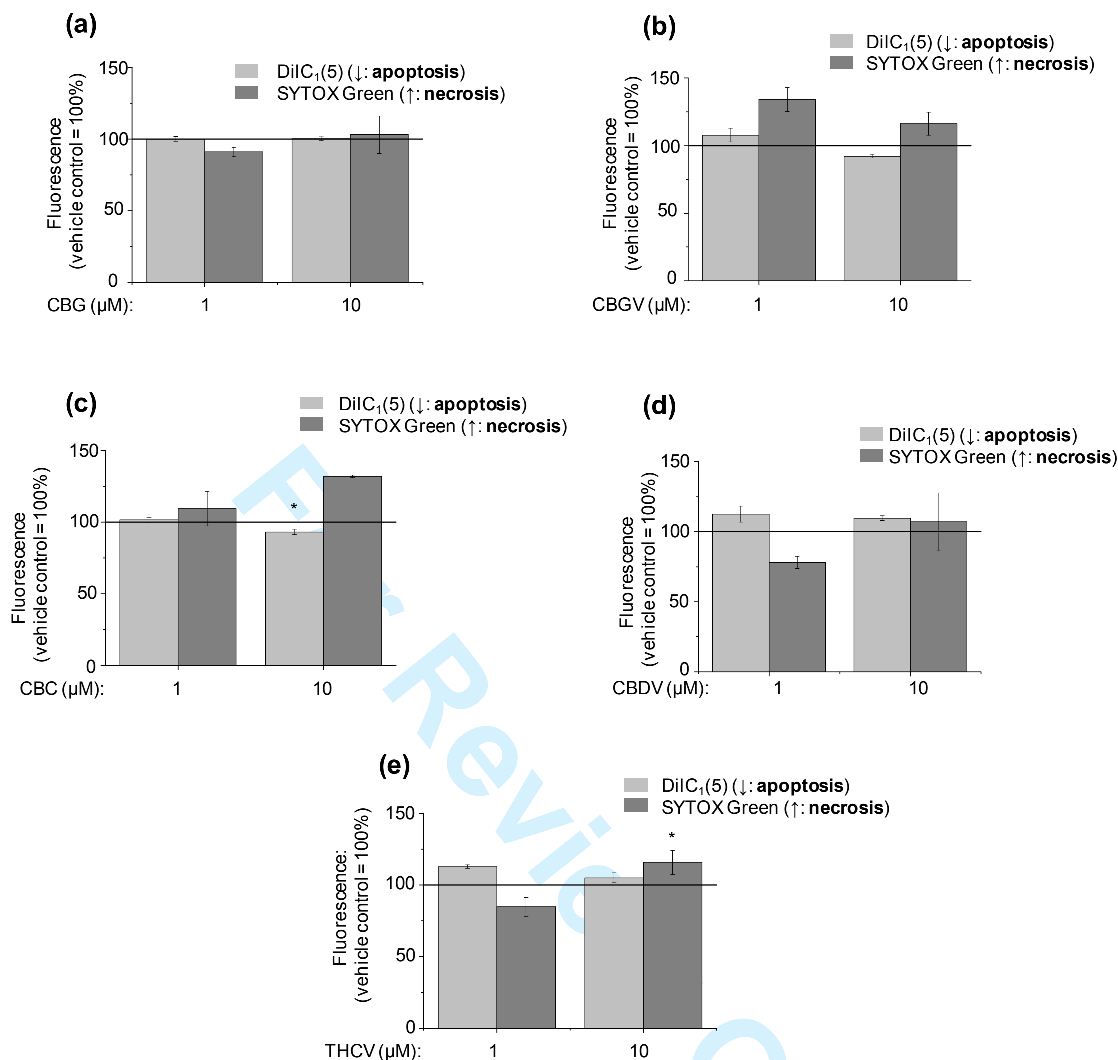

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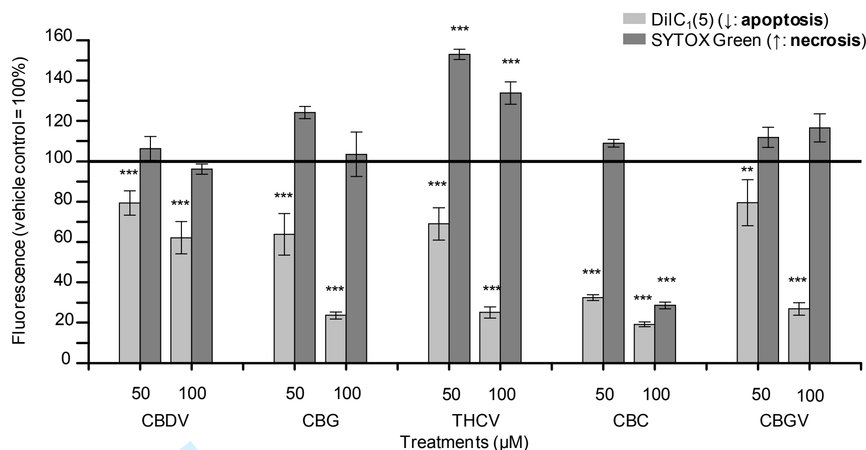
Supplementary Figure S4 *Up to 10 µM, the investigated pCBs induced only minor alterations in the ratio of apoptotic or necrotic cells following 24-hr treatments*

(**a-e**) Apoptotic and necrotic cell death of SZ95 sebocytes was investigated by $DiIC_1(5)$-SYTOX Green double labelling following the indicated 24-hr treatments. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of four independent determinations. * marks significant (P<0.05) differences compared to the vehicle control. One additional experiment yielded similar results. CBC: (-)-cannabichromene; CBDV: (-)-cannabidivarin; CBG: (-)-cannabigerol; CBGV: (-)-cannabigerovarin; THCV: (-)-Δ^9-tetrahydrocannabivarin.



Supplementary Figure S5 *High concentrations of the pCBs induced apoptosis-dominated cell death*

Apoptotic and necrotic cell death of SZ95 sebocytes was investigated by $DiIC_1(5)$-SYTOX Green double labelling assays following the indicated 24-hr treatments. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of four independent determinations. One additional experiment yielded similar results. ** and *** mark significant ($P<0.01$ or 0.001, respectively) differences compared to the vehicle control. CBC: (-)-cannabichromene; CBDV: (-)-cannabidivarin; CBG: (-)-cannabigerol; CBGV: (-)-cannabigerovarin; THCV: (-)-Δ^9-tetrahydrocannabivarin.

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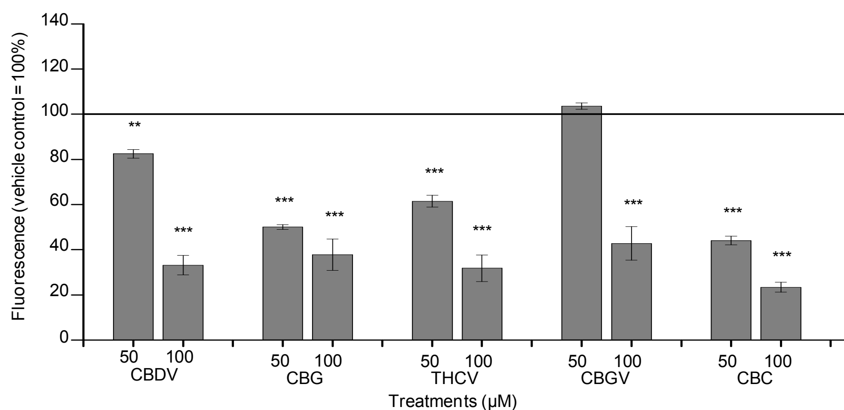

Supplementary Figure S6 *High concentrations of the pCBs suppressed sebaceous lipid synthesis*

Sebaceous lipid production of SZ95 sebocytes was assessed by Nile Red staining following 24-hr treatments. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of four independent determinations. One additional experiment yielded similar results. ** and *** mark significant (*P*<0.01 or 0.001, respectively) differences compared to the vehicle control. CBC: (-)-cannabichromene; CBDV: (-)-cannabidivarin; CBG: (-)-cannabigerol; CBGV: (-)-cannabigerovarin; THCV: (-)-Δ^9-tetrahydrocannabivarin.

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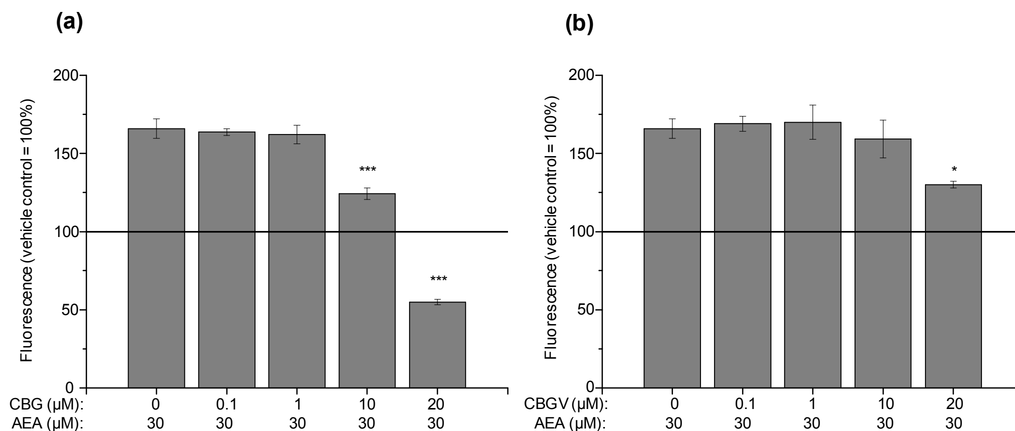

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Supplementary Figure S7 *CBG and CBGV reduce AEA-induced lipogenesis of human sebocytes*

Sebaceous lipid production of SZ95 sebocytes was assessed by Nile Red staining following 48-hr treatments. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of 4-8 independent determinations. One additional experiment yielded similar results. * and *** mark significant (*P*<0.05 or 0.001, respectively) differences compared to the AEA-treated group. AEA: anandamide; CBG: (-)-cannabigerol; CBGV: (-)-cannabigerovarin.

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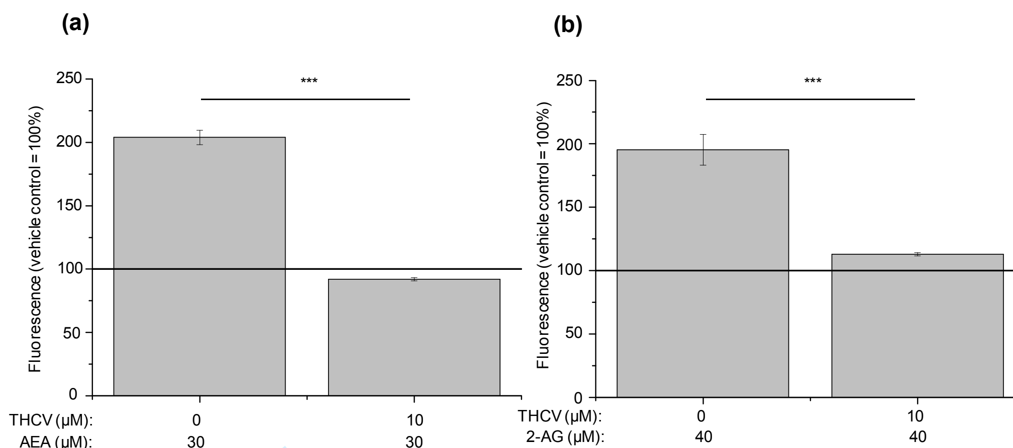

Supplementary Figure S8 *THCV exerts universal lipostatic effects*

(**a-b**) Sebaceous lipid production of SZ95 sebocytes was monitored by Nile Red staining following 48-hr treatments. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of 4 independent determinations. One additional experiment yielded similar results. *** marks significant (P<0.001) differences as indicated. 2-AG: 2-arachidonoylglycerol; AEA: anandamide; THCV: (-)-Δ^9-tetrahydrocannabivarin.

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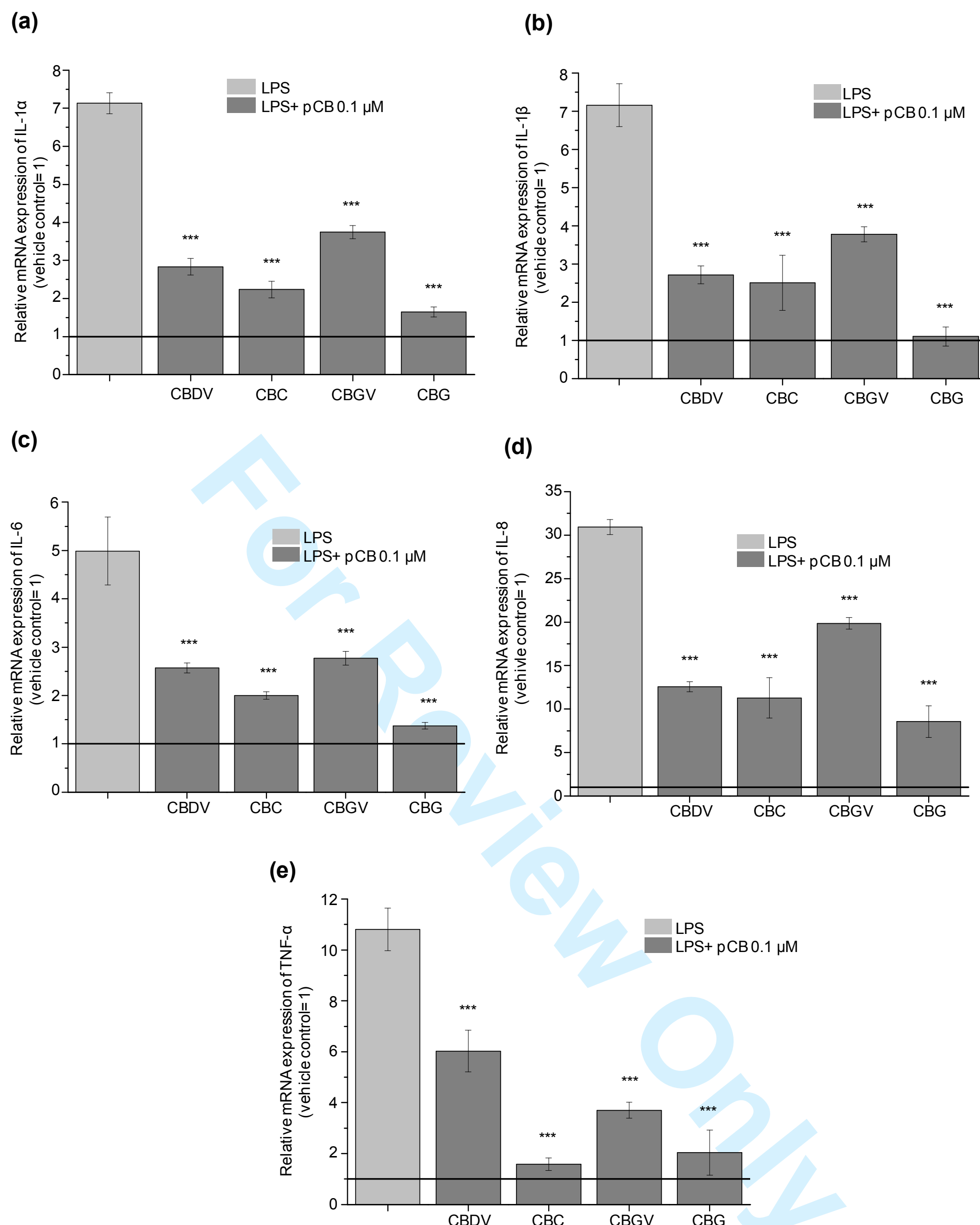

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Supplementary Figure S9 *pCBs exert remarkable anti-inflammatory actions in human sebocytes*

(**a-e**) Q-PCR analyses of SZ95 sebocytes following the indicated 3-hr simultaneous treatments. Data are presented by using ΔΔCT method regarding 18S RNA-normalized mRNA expressions of the vehicle control as 1 (solid line). Data are expressed as mean±SD of 2-3 determinations (**a**: *IL-1α*; **b**: *IL-1β*; **c**: *IL-6*; **d**: *IL-8*; **e**: *TNF-α*). One additional experiment yielded similar results. *** marks significant

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(P<0.001) differences compared to the LPS-treated group. CBC: (-)-cannabichromene; CBDV: (-)-cannabidivarin; CBG: (-)-cannabigerol; CBGV: (-)-cannabigerovarin; pCB: phytocannabinoid; THCV: (-)-Δ^9-tetrahydrocannabivarin.

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