SUPPLEMENTARY MATERIAL

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

Attila Oláh[1*], Arnold Markovics[1*], Judit Szabó-Papp[1], Pálma Tímea Szabó[1], Colin Stott[2], Christos C. Zouboulis[3], Tamás Bíró[1,4]

[1]DE-MTA "Lendület" Cellular Physiology Research Group, Department of Physiology, Faculty of Medicine, University of Debrecen, Debrecen, H-4032, Hungary; [2]GW Pharmaceuticals, Cambridge, UK; [3]Departments of Dermatology, Venereology, Allergology and Immunology, Dessau Medical Center, Dessau, 06847, Germany; [4]Department of Immunology, Faculty of Medicine, University of Debrecen, Debrecen, H-4032, Hungary

[*]These authors contributed equally to this publication.

Correspondence should be addressed to Tamás Bíró (University of Debrecen, Nagyerdei krt. 98. Debrecen, H-4032, Hungary; e-mail: biro.tamas@med.unideb.hu; phone: +3652-255-575; fax: +3652-255-116).

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).

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

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 CB_1 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.

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™ $DilC_1(5)$ Assay Kit (Life Technologies Hungary Ltd.). Cells (20,000 cells/well) were cultured in black 96-well plates with clear bottom (Greiner Bio One) in quadruplicates and were treated as indicated for 24 hrs. After removal of supernatants, cells were incubated for 30 minutes with $DilC_1(5)$ working solution (50 µl/well), then washed with PBS, and the fluorescence of $DilC_1(5)$ 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

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 black 96-well plates with clear bottom (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**.

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).

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,

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

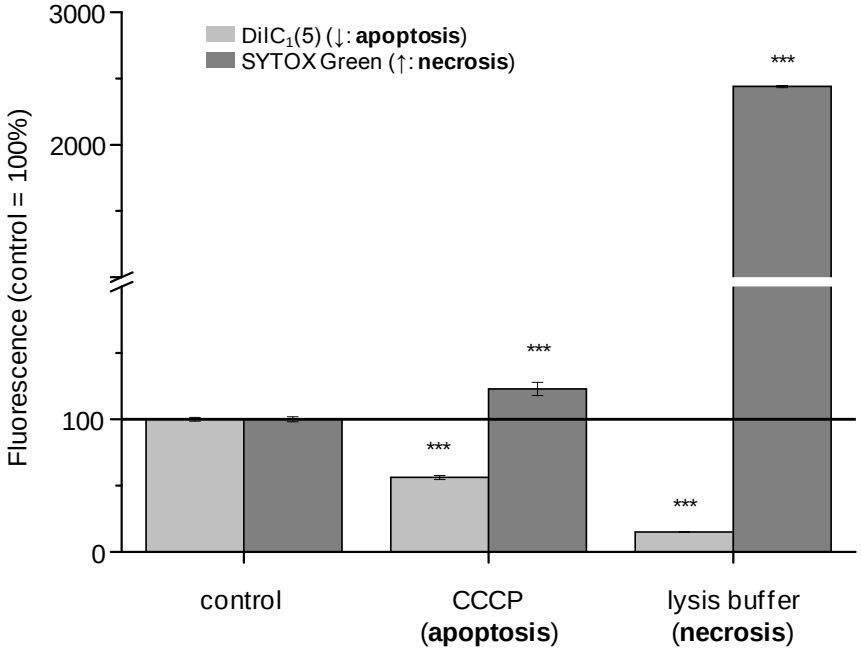
As discussed above, pCBs are well-known to influence the activity of a wide-array of cellular targets, including receptors (CB_1, CB_2, 5-HT_{1A}, adenosine A_{2A}, GPR55, μ opioid) enzymes (FAAH, phospholipase A2, cyclooxygenase, lipoxygenase or certain cytochrome P450 enzymes, etc.), transporters (EMT, 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).

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).

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.

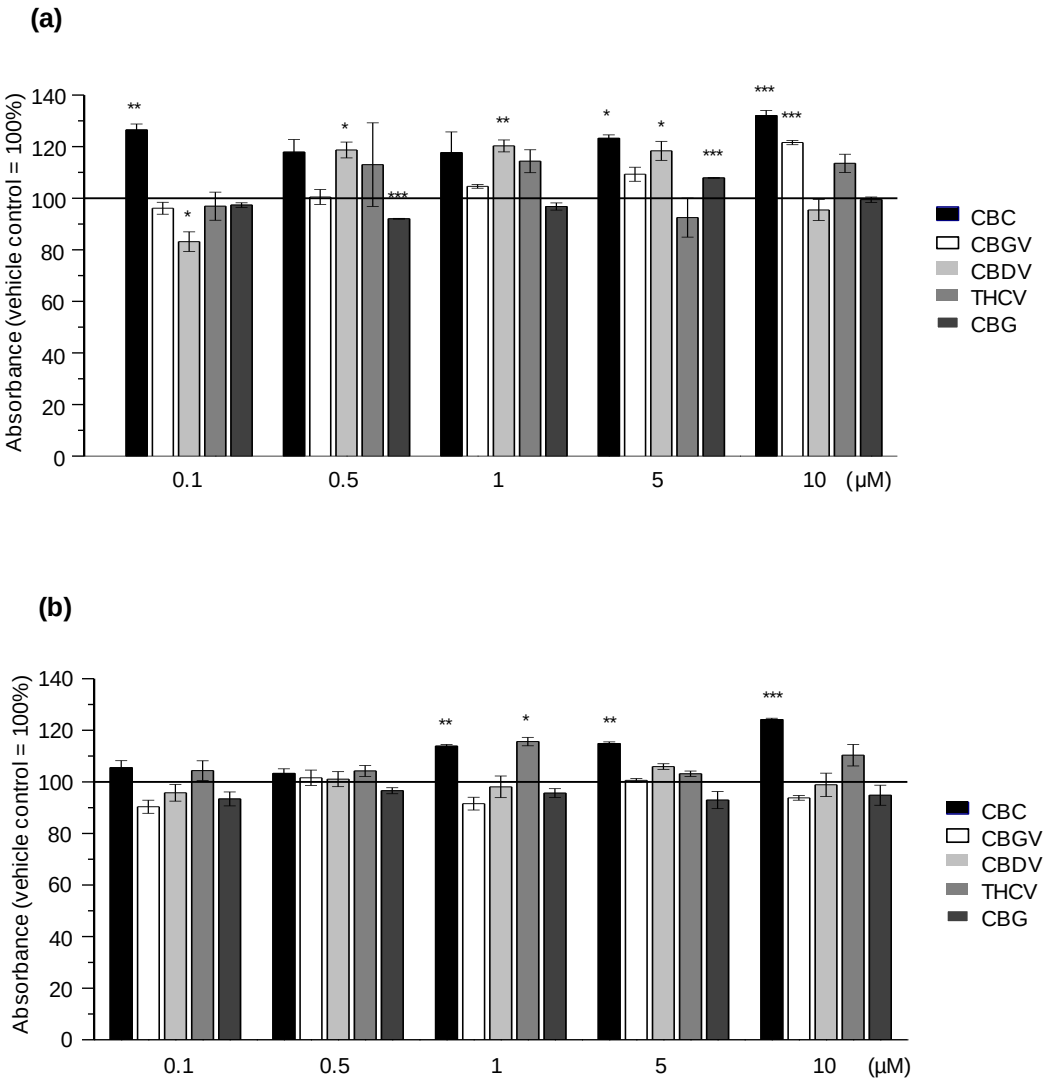
On the other hand, we should not forget about the fact that, besides the sebocytes, pCBs may reach other cutaneous cells (epidermal keratinocytes, melanocytes, Langerhans cells, fibroblasts, cells of the hair follicles, etc.) as well. Although, as mentioned in the **Discussion & Conclusions section** 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.

Supplementary Figures



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

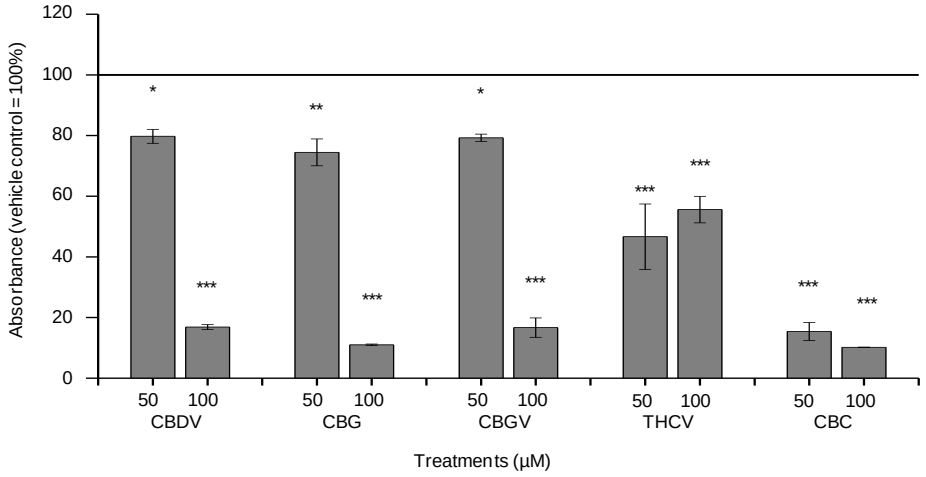
Cell death of SZ95 sebocytes was investigated by combined fluorescent $DilC_1(5)$-SYTOX Green labelling. As apoptosis positive control, carbonyl cyanide m-chlorophenyl hydrazone (CCCP; a well-known un-coupler of the mitochondrial inner membrane) was applied (1:200 for 30 min), whereas necrosis was induced 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 $DilC_1(5)$ 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 $DilC_1(5)$ 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.



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.

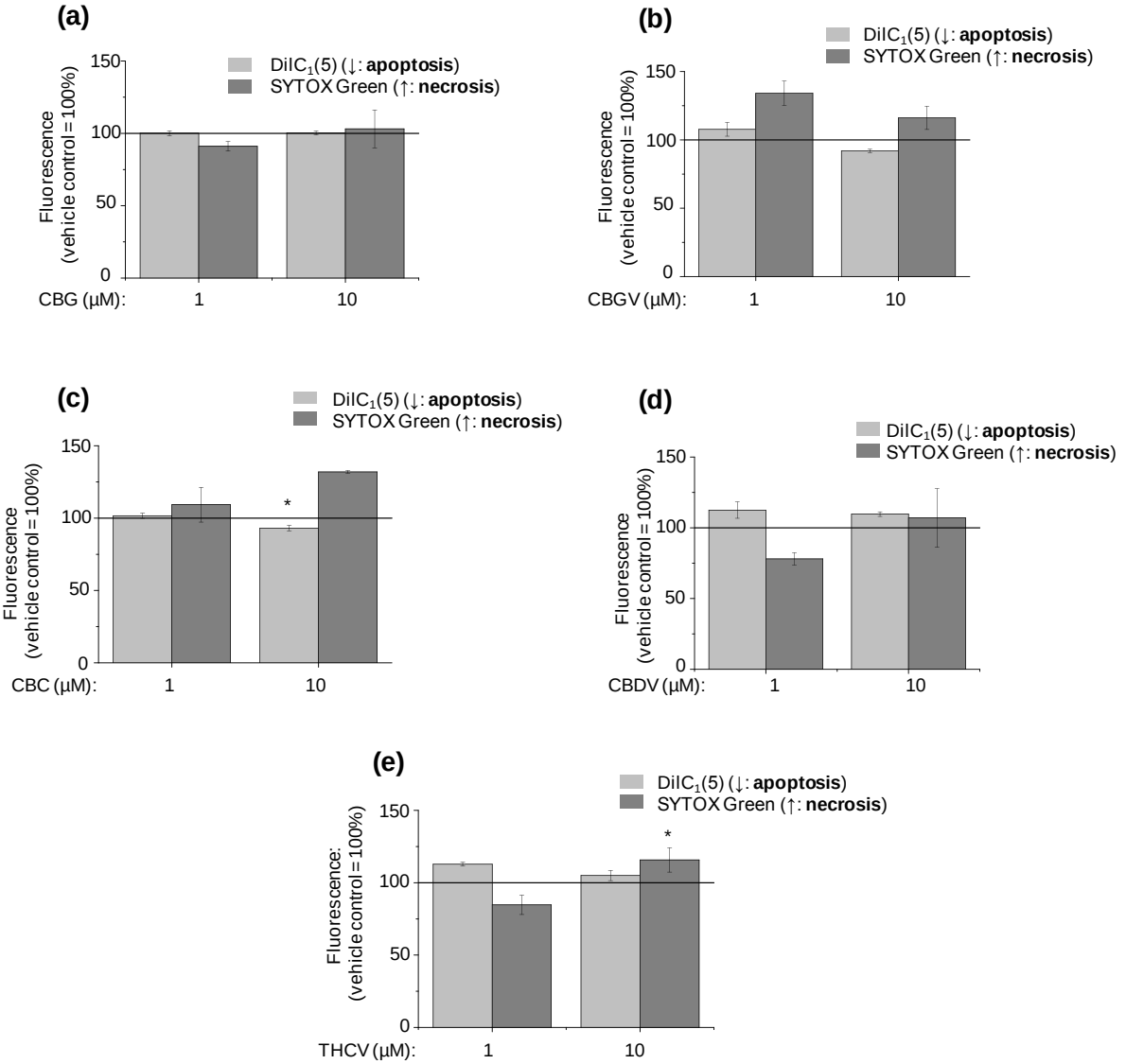
CBC: (-)-cannabichromene; CBDV: (-)-cannabidivarin; CBG: (-)-cannabigerol; CBGV: (-)-cannabigerovarin; THCV: (-)-Δ^9-tetrahydrocannabivarin.



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.

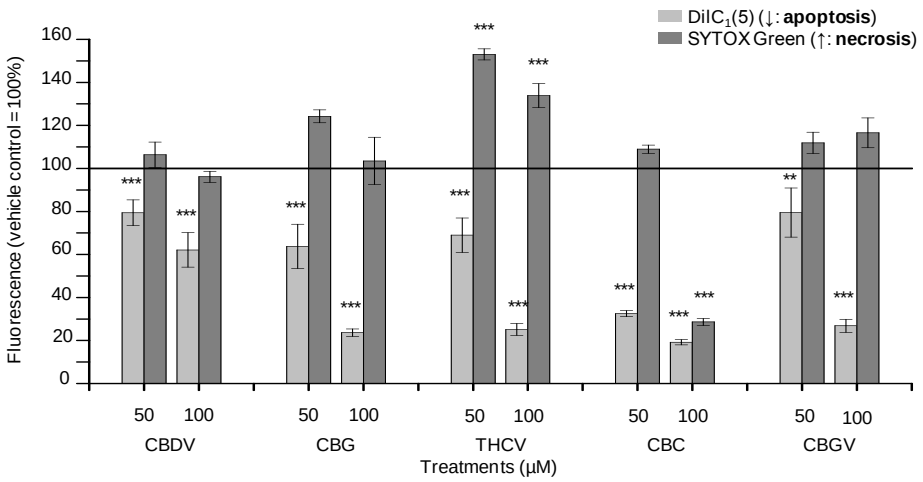
CBC: (-)-cannabichromene; CBDV: (-)-cannabidivarin; CBG: (-)-cannabigerol; CBGV: (-)-cannabigerovarin; THCV: (-)-Δ^9-tetrahydrocannabivarin.



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.

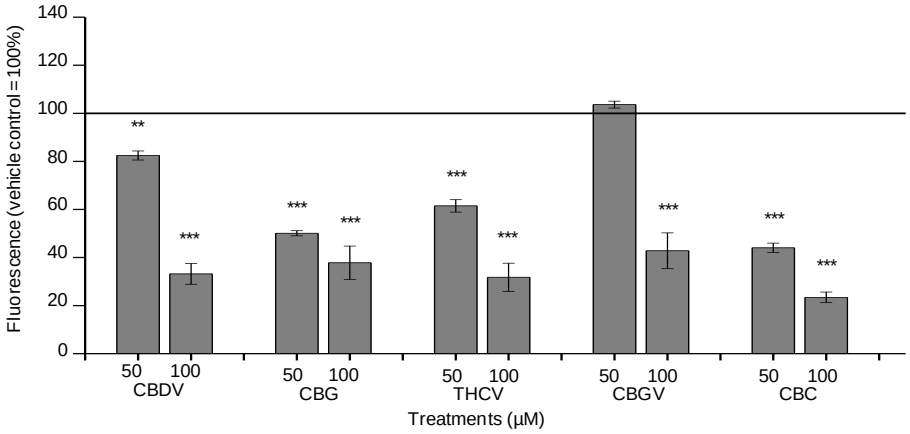
CBC: (-)-cannabichromene; CBDV: (-)-cannabidivarin; CBG: (-)-cannabigerol; CBGV: (-)-cannabigerovarin; THCV: (-)-Δ^9-tetrahydrocannabivarin.



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.

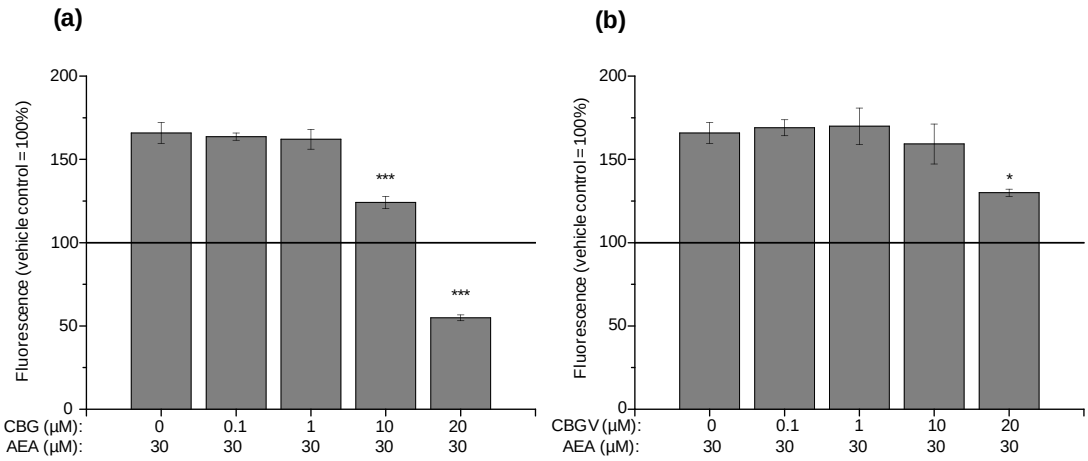
CBC: (-)-cannabichromene; CBDV: (-)-cannabidivarin; CBG: (-)-cannabigerol; CBGV: (-)-cannabigerovarin; THCV: (-)-Δ^9-tetrahydrocannabivarin.



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.

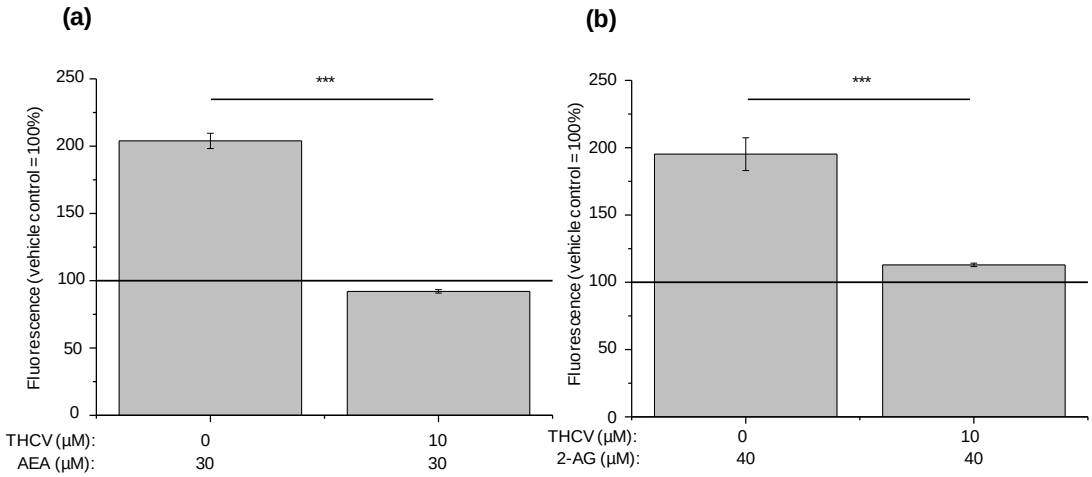
CBC: (-)-cannabichromene; CBDV: (-)-cannabidivarin; CBG: (-)-cannabigerol; CBGV: (-)-cannabigerovarin; THCV: (-)-Δ^9-tetrahydrocannabivarin.



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.

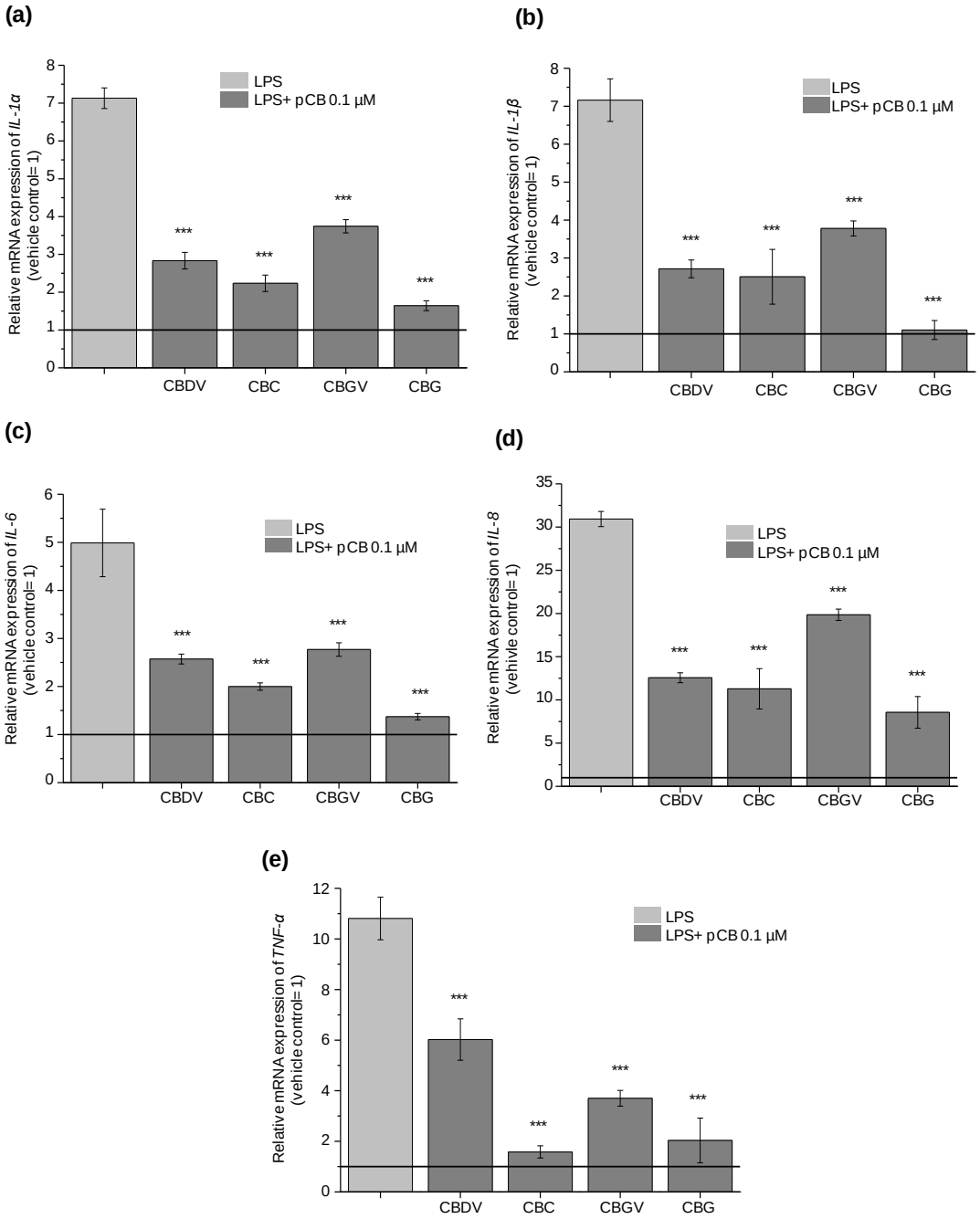


Supplementary Figure S8 *THCV exerts universal lipostatic effects*

(**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.



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

(**a-e**) qPCR 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

(*P*<0.001) differences compared to the LPS-treated group.

CBC: (-)-cannabichromene; CBDV: (-)-cannabidivarin; CBG: (-)-cannabigerol;

CBGV: (-)-cannabigerovarin; pCB: phytocannabinoid;

THCV: (-)-Δ^9-tetrahydrocannabivarin.

Supplementary References

s1 Di Marzo V. Targeting the endocannabinoid system: to enhance or reduce? Nat Rev Drug Discov 2008: **7**: 438–455.

s2 Pertwee R G. Endocannabinoids and Their Pharmacological Actions. Handb Exp Pharmacol 2015: **231:** 1–37.

s3 Caterina M J. TRP channel cannabinoid receptors in skin sensation, homeostasis, and inflammation. ACS Chem Neurosci 2014: **5**: 1107–1116.

s4 Sugawara K, Bíró T, Tsuruta D *et al.* Endocannabinoids limit excessive mast cell maturation and activation in human skin. J Allergy Clin Immunol 2012: **129**: 726–738.e8.

s5 Bíró T, Tóth B I, Haskó G *et al.* The endocannabinoid system of the skin in health and disease: novel perspectives and therapeutic opportunities. Trends Pharmacol Sci 2009: **30**: 411–420.

s6 Thomas A, Stevenson L A, Wease K N *et al.* Evidence that the plant cannabinoid Delta9-tetrahydrocannabivarin is a cannabinoid CB1 and CB2 receptor antagonist. Br J Pharmacol 2005: **146**: 917–926.

s7 Pertwee R G. The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin. Br J Pharmacol 2008: **153**: 199–215.

s8 Rock E M, Sticht M A, Duncan M *et al.* Evaluation of the potential of the phytocannabinoids, cannabidivarin (CBDV) and Δ(9) -tetrahydrocannabivarin (THCV), to produce CB1 receptor inverse agonism symptoms of nausea in rats. Br J Pharmacol 2013: **170**: 671–678.

s9 Rosenthaler S, Pöhn B, Kolmanz C *et al.* Differences in receptor binding affinity of several phytocannabinoids do not explain their effects on neural cell cultures. Neurotoxicol Teratol 2014: **46**: 49–56.

s10 Rosenthaler S, Pöhn B, Kolmanz C *et al.* Corrigendum to “Differences in receptor binding affinity of several phytocannabinoids do not explain their effects on neural cell cultures” [NTT. 46C (2014) 49–56] [Internet]. Neurotoxicology and Teratology [cited 2016 Jan 14]
DOI: http://www.sciencedirect.com/science/article/pii/S0892036215002469

s11 Pucci M, Rapino C, Francesco A Di *et al.* Epigenetic control of skin differentiation genes by phytocannabinoids. Br J Pharmacol 2013: **170**: 581–591.

s12 Appendino G, Gibbons S, Giana A *et al.* Antibacterial cannabinoids from Cannabis sativa: a structure-activity study. J Nat Prod 2008: **71**: 1427–1430.

s13 Akopian A N, Ruparel N B, Jeske N A *et al.* Role of ionotropic cannabinoid receptors in peripheral antinociception and antihyperalgesia. Trends Pharmacol Sci 2009: **30**: 79–84.

s14 Bisogno T, Hanus L, De Petrocellis L *et al.* Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. Br J Pharmacol 2001: **134**: 845–852.

s15 Carrier E J, Auchampach J A, Hillard C J. Inhibition of an equilibrative nucleoside transporter by cannabidiol: a mechanism of cannabinoid immunosuppression. Proc Natl Acad Sci USA 2006: **103**: 7895–7900.

s16 Kathmann M, Flau K, Redmer A *et al.* Cannabidiol is an allosteric modulator at mu- and delta-opioid receptors. Naunyn Schmiedebergs Arch Pharmacol 2006: **372**: 354–361.

s17 Laprairie R B, Bagher A M, Kelly M E M *et al.* Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. Br J Pharmacol 2015: **172**: 4790–4805.

s18 Qin N, Neeper M P, Liu Y *et al.* TRPV2 is activated by cannabidiol and mediates CGRP release in cultured rat dorsal root ganglion neurons. J Neurosci 2008: **28**: 6231–6238.

s19 Rimmerman N, Ben-Hail D, Porat Z *et al.* Direct modulation of the outer mitochondrial membrane channel, voltage-dependent anion channel 1 (VDAC1) by cannabidiol: a novel mechanism for cannabinoid-induced cell death. Cell Death Dis 2013: **4**: e949.

s20 Ross R A. The enigmatic pharmacology of GPR55. Trends Pharmacol Sci 2009: **30**: 156–163.

s21 Russo E B, Burnett A, Hall B *et al.* Agonistic properties of cannabidiol at 5-HT1a receptors. Neurochem Res 2005: **30**: 1037–1043.

s22 Straus S E. Immunoactive cannabinoids: therapeutic prospects for marijuana constituents. Proc Natl Acad Sci USA 2000: **97**: 9363–9364.

s23 Stanley C P, Hind W H, Tufarelli C *et al.* Cannabidiol causes endothelium-dependent vasorelaxation of human mesenteric arteries via CB1 activation. Cardiovasc Res 2015: **107**: 568–578.

s24 Brunton LL, Chabner BA, Knollman BC, eds. Goodman & Gilman's The Pharmacological Basis of Therapeutics. 12th ed., New York: McGrawHill, 2011.

s25 Katzung BG, Masters SG, Trevor AJ, eds. Basic & Clinical Pharmacology. 12th ed., New York: McGraw-Hill, 2012.