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Endocannabinoid tone regulates human sebocyte biology

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Short title: Sebocytes' endocannabinoid system

Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, arachidonoylethanolamide (a.k.a. anandamide); DAGL, diacylglycerol lipase; eCB, endocannabinoid; $EtNH_2$,

ethanolamine; ECS, endocannabinoid system; EMT, endocannabinoid membrane transporter; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; NAPE-PLD, *N*-acyl phosphatidylethanolamine-specific phospholipase D; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; SG, sebaceous gland; SLP, sebaceous lipid production

ABSTRACT

We have previously shown that i) endocannabinoids (eCB; e.g. anandamide [AEA]) are involved in the maintenance of homeostatic sebaceous lipid production (SLP) in human sebaceous glands (SG); and ii) eCB treatment dramatically increases SLP. Here, we aimed to investigate the expression of the major eCB synthesizing and degrading enzymes, and to study the effects of eCB uptake inhibitors on human SZ95 sebocytes, thus exploring the role of the putative eCB membrane transporter (EMT), which has been hypothesized to facilitate the cellular uptake and subsequent degradation of eCBs.

We found that the major eCB synthesizing (NAPE-PLD, DAGLα and -β) and degrading (FAAH, MAGL) enzymes are expressed in SZ95 sebocytes, and also in SGs (except for DAGLα, whose staining was dubious in histological preparations). Interestingly, eCB uptake-inhibition with VDM11 induced a moderate increase in SLP, and also elevated the levels of various eCBs and related acylethanolamides. Finally, we found that VDM11 was able to interfere with the pro-inflammatory action of the Toll-like receptor 4 activator lipopolysaccharide.

Collectively, our data suggest that inhibition of eCB uptake exerts anti-inflammatory actions and elevates both SLP and eCB levels; thus, these inhibitors might be beneficial in cutaneous inflammatory conditions accompanied by dry skin.

INTRODUCTION

Sebaceous glands (SG) are important players and regulators of the human skin homeostasis. Indeed, besides their obvious function, i.e. production of the lipid-rich sebum, and thereby contribution to the cutaneous lipid barrier and thermoregulation, they also play a role in the endocrine and immune systems of the skin, and serve as stem cell reservoirs as well (Dajnoki et al. 2017; Lupi 2008; Porter 2001; Tóth et al. 2011; Zouboulis et al. 2014; Zouboulis et al. 2008). Importantly, their clinical significance is also remarkably high, since overproduction and pathologically altered composition of sebum in seborrhea is a key step in the pathogenesis of acne, one of the most prevalent human skin diseases (Kurokawa et al. 2009; Tóth et al. 2011; Zouboulis et al. 2014; Zouboulis et al. 2008). On the other hand, lack of sufficient sebum production in adulthood may contribute to dry-skin syndrome, xerosis or even skin ageing and atopic dermatitis (AD) (Kim et al. 2014; Mischo et al. 2014; Shi et al. 2015; Zampeli et al. 2012; Zouboulis and Boschnakow 2001). Moreover, unique composition of sebum is thought to play an important role in regulating the growth of the cutaneous microbiota by restricting the unwanted microbes, and promoting the preferred ones, thus making homeostatic SG functions important orchestrators of skin-microbiota cross-talk (Pappas 2009). Hence, via the subsequent pathological alterations in the cutaneous microbiota, disorders of sebaceous lipid production (SLP) may contribute to the pathogenesis of several diseases, including AD (Shi et al. 2015). Therefore, a better understanding of (dys)regulation of SG biology, and identification of additional regulators of homeostatic SLP are clinically relevant topics of investigative dermatology. Unfortunately, human SGs are challenging to study, since primary sebocytes cannot be kept in culture for more than a few passages, and there are no adequate animal model systems to assess the

whole complexity of the human SG biology (Tóth et al. 2011; Zouboulis et al. 2014; Zouboulis et al. 2008). Therefore, within the scope of the current study, we used the human immortalized SZ95 sebocyte cell line (Zouboulis et al. 1999), which is a widely accepted model system to study human SG functions *in vitro* (Tóth et al. 2011; Zouboulis et al. 2014; Zouboulis et al. 2008).

Interestingly, a growing body of evidence suggests that SGs are not only "innocent" targets of the complex cutaneous neuroendocrine system (Slominski 2005; Slominski and Wortsman 2000), but also its master regulators by producing several hormones and paracrine signal molecules (e.g. androgen hormones, corticotropin-releasing hormone, several adipokines, etc.) (Kovács et al. 2016; Tóth et al. 2011; Zouboulis et al. 2014; Zouboulis et al. 2008). Furthermore, we have previously shown that, besides the aforementioned regulators, human SGs are also important players in the endocannabinoid system (ECS) of the skin (Dobrosi et al. 2008; Oláh and Bíró 2017).

The ECS is a complex signaling system, comprising endogenous ligands (i.e. the "endocannabinoids" [eCB], e.g. arachidonoylethanolamide [a.k.a. anandamide, AEA], 2-arachidonoylglycerol [2-AG]), receptors (e.g. CB_1 and CB_2, etc.), as well as multiple enzymes involved in the synthesis (e.g. *N*-acyl phosphatidylethanolamine-specific phospholipase D [NAPE-PLD], diacylglycerol lipase [DAGL]-α and –β, etc.) and degradation (e.g. fatty acid amide hydrolase [FAAH], monoacylglycerol lipase [MAGL]) of the eCBs and related non-eCB mediators (such as monoacylglycerols and acylethanolamides, like palmitoylethanolamide [PEA], oleoylethanolamide [OEA],

etc.). Moreover, a putative eCB membrane transport mechanism (usually referred to as putative eCB membrane transporter [EMT]), postulated to facilitate cellular uptake and release of AEA and 2-AG also belongs to this system (Chicca et al. 2012; Ligresti et al. 2016; Maccarrone 2017; Maccarrone et al. 2015; Nicolussi and Gertsch 2015; Solymosi and Köfalvi 2016). Importantly, we have previously shown that human SGs are capable of producing the major eCBs (namely AEA and 2-AG), and we could also demonstrate that (in line with the early data of the Ständer group) SGs express CB_1 (mostly in differentiated cells) and CB_2 receptors (predominantly in proliferating, basal layer sebocytes) (Dobrosi et al. 2008; Ständer et al. 2005). Moreover, we found that locally produced eCBs, acting through a CB_2-coupled signaling pathway, are key players in the maintenance of the homeostatic SLP, since selective gene silencing of CB_2 significantly reduced basal SLP of SZ95 sebocytes, whereas eCB-treatment of the cells led to greatly increased lipogenesis (Dobrosi et al. 2008). In this way, we provided to our knowledge previously unreported evidence that human SGs possess a functionally active ECS and that treatment of human sebocytes with exogenously administered AEA or 2-AG dramatically increases SLP. However, we did not have any data about either the expression of the enzyme apparatus involved in the synthesis and degradation of the eCBs, or the role of the local eCB tone created by these enzymes in human sebocytes. Thus, within the confines of the current, highly focused study, we aimed to explore the expression of the major members of the ECS *in vitro* in human sebocytes, as well as *in situ* in human skin, and we also wanted to investigate if pharmacological modulation of eCB homeostasis was indeed able to regulate SLP.

RESULTS

Major enzymes of the eCB metabolism are expressed in cultured human sebocytes as well as *in situ* in human SGs

First, by using human, immortalized SZ95 sebocytes, we investigated expression of the major enzymes involved in the synthesis and degradation of AEA (NAPE-PLD and FAAH, respectively) and 2-AG (DAGLα and –β and MAGL, respectively). We found that, irrespective of the confluence level of the cells, all the above mentioned enzymes were expressed in human sebocytes both at the mRNA (Q-PCR) and protein (Western blot) levels (**Supplementary Figure S1a-d**). In order to further confirm these results, we also investigated their expression in human skin samples (appropriately labeled positive controls are shown on **Supplementary Figure S2**). Importantly, with the sole exception of DAGLα, which exhibited questionable expression when compared to the endogenous positive control sweat glands (Czifra et al. 2012), our findings nicely confirmed our *in vitro* data about the expression of the above enzymes in human SGs *in situ* (**Figure 1**).

Sebocytes exhibit a pharmacologically inhibitable eCB uptake process

Next, we assessed if pharmacologically inhibitable AEA uptake by the putative EMT (Chicca et al. 2012; Nicolussi and Gertsch 2015) was observable in these cells. By monitoring the uptake of radiolabeled [^{3}H]AEA by the cells, we found that acute inhibition (15 min, 10 µM) of AEA cellular uptake using the reference eCB transport inhibitor UCM707 (López-Rodríguez et al. 2003; Rau et al. 2016) was able to significantly alter the eCB uptake process. Indeed, UCM707 reduced the intracellular

[^{3}H]AEA signal, which was accompanied by a significant increase in the extracellular [^{3}H]AEA levels compared to vehicle control. As a consequence of [^{3}H]AEA uptake inhibition, the overall hydrolysis of [^{3}H]AEA by FAAH to arachidonic acid and [^{3}H]ethanolamine ([^{3}H]$EtNH_2$) was reduced (**Figure 2a**). Theoretically, a decrease of [^{3}H]AEA uptake and [^{3}H]$EtNH_2$ levels could be explained not only by the inhibition of the eCB transport process, but also by non-specific inhibition of FAAH resulting in a reduced driving force for [^{3}H]AEA uptake (Chicca et al. 2012; Nicolussi et al. 2014a). To exclude this possibility, we assessed how two different EMT-inhibitors (namely UCM707 and VDM11; both widely used to abrogate cellular uptake of eCBs) (De Petrocellis et al. 2000; López-Rodríguez et al. 2003), influence FAAH activity in SZ95 sebocytes, in comparison with the reference FAAH-inhibitor URB597 (Mor et al. 2004). Importantly, we found that SZ95 sebocytes exhibit very low constitutive FAAH activity (2.32±0.25 pmol/min/mg protein; n=10), and that, unlike URB597, neither UCM707, nor VDM11 exerted substantial FAAH-inhibition (IC_{50} values were above 25 µM for both EMT-inhibitors, whereas IC_{50} of URB597 was below 100 nM) (**Supplementary Table S1**). Taken together, these findings suggest that in human sebocytes eCB uptake can be inhibited by VDM11 most likely through a FAAH independent manner.

EMT plays a role in the degradation of eCBs in human sebocytes

Next, we investigated the eCB transport process using one of the aforementioned EMT-inhibitors, VDM11 (De Petrocellis et al. 2000). Cells were treated with this compound or vehicle for 24 hours and eCB content of the samples was analyzed by LC-MS. We

found that VDM11 treatment significantly increased AEA content of the samples (**Figure 2b**), whereas elevation of 2-AG concentration did not reach statistical significance (**Figure 2c**). Besides the two major eCBs, we also investigated the presence and potential alterations of two ECS-related acylethanolamides, i.e. PEA and OEA. Interestingly, we found that treatment by VDM11 tended to increase PEA, and significantly elevated OEA concentrations (**Figure 2d-e**). Collectively, these data provided evidence that administration of VDM11 can increase the levels of certain eCBs and eCB-like mediators in human sebocytes. These results hold out the promise that VDM11 might therefore promote homeostatic eCB and eCB-like mediator signaling in these cells.

Administration of eCB membrane transport inhibitors mimics lipogenic actions of direct eCB-treatment, whereas selective FAAH-inhibition does not influence SLP

It is well-described that direct eCB-treatment of the sebocytes results in a dramatically increased SLP (Dobrosi et al. 2008; Oláh et al. 2016b; Oláh et al. 2014). Thus, next we wanted to assess how treatment with eCB uptake inhibitors influences SG's functions. Using non-cytotoxic concentrations (determined by MTT-assay; **Supplementary Figure S3**) of the aforementioned VDM11, we explored its effect on SLP. By using fluorescent Nile Red staining, we found that its low micromolar concentrations induced a moderate, but significant increase in the SLP following 48-hr treatments (**Figure 3a**). Importantly, repetition of the experiment by using AM404 (another well-known AEA uptake inhibitor) (Beltramo et al. 1997; Nicolussi and Gertsch 2015) yielded very similar results (**Supplementary Figure S4a-b**). Intriguingly, although both VDM11

and AM404 mimicked the lipogenic actions of the prototypic eCB AEA (Dobrosi et al. 2008), their efficiencies at elevating SLP were far exceeded by direct AEA-treatment (**Figure 3a**).

As discussed above, several lines of evidence demonstrate that eCB uptake inhibitors may concentration-dependently inhibit not only the putative EMT, but FAAH as well (Beltramo et al. 1997; Chicca et al. 2017; Nicolussi and Gertsch 2015). Although our current measurements on sebocytes' FAAH-activity already provided strong evidence that i) SZ95 sebocytes exhibit very low constitutive FAAH activity; and ii) in SZ95 sebocytes, both UCM707 and VDM11 can be used at 10 μM without the risk of having substantial impact on FAAH activity (**Supplementary Table S1**), we also intended to investigate biological effects of the selective reference FAAH-inhibitor URB597 (Mor et al. 2004). Interestingly, we found that administration of URB597 led to different cell physiology outcome than application of VDM11 and AM404. Indeed, our data showed that non-cytotoxic concentrations (MTT-assay; **Supplementary Figure S5a**) of URB597 did not influence SLP (48-hr treatments; **Supplementary Figure S5b**) as compared to the vehicle control group, indicating that abrogation of eCB uptake, but not inhibition of their FAAH-mediated intracellular degradation, leads to the elevation of the SLP. It is also important to note that such lack of effect by URB597 can most likely be ascribed to the aforementioned very low levels of FAAH activity in SZ95 sebocytes.

Lipogenic action of direct AEA treatment is not further increased by co-administration of VDM11

Next, we intended to assess how co-administration of AEA and VDM11 affect SLP. Interestingly, we found that VDM11 was unable to further promote the AEA-induced, already elevated SLP (**Supplementary Figure S6**) of human sebocytes, suggesting that the pro-lipogenic eCB signaling activated by 30 μM AEA is exhaustive, and has no further "reserve capacity".

Up to 10 μM, VDM11 does not induce apoptosis of human sebocytes

Elevation of SLP is the hallmark of sebocyte differentiation, which is usually followed by programmed cell death (Dobrosi et al. 2008; Fischer et al. 2017; Tóth et al. 2011; Zouboulis et al. 2014; Zouboulis et al. 2008). Since VDM11 was shown to moderately promote SLP (**Figure 3a**; **Supplementary Figure S4b**), we also wanted to know if it induced early apoptotic processes. Importantly, we found that, although the most effective lipogenic concentration of VDM11 tended to decrease mitochondrial membrane potential in course of 48-hr treatments (**Supplementary Figure S7**), this did not reach the level of significance, suggesting that within the studied time-frame, it may indeed be devoid of obvious pro-apoptotic effects.

VDM11 suppresses lipopolysaccharide (LPS)-induced pro-inflammatory cytokine expression of human sebocytes

From a clinical point-of-view, induction of a moderate (not seborrheic or acnegenic) increase in the homeostatic SLP would be highly desirable in the treatment of skin dryness (Kim et al. 2014; Mischo et al. 2014; Shi et al. 2015; Zampeli et al. 2012;

Zouboulis and Boschnakow 2001). Thus, our above results suggest that administration of VDM11 (and probably other EMT-inhibitors too) may possess beneficial effects in such conditions. Considering that skin dryness is frequently accompanied by cutaneous inflammation (e.g. in the case of AD) (Peng and Novak 2015; Sugiura et al. 2014), we finally investigated how VDM11 affects immune properties of human sebocytes. To this end, we applied lipopolysaccharide (LPS; 5 μg/ml; 3-hr treatments) (Oláh et al. 2016b) to induce pro-inflammatory response. Importantly, co-administration of VDM11 (10 μM) efficiently suppressed LPS-induced expression of *interleukin* (*IL*)*-1α*, *IL-1β*, *IL-6*, *IL-8* and *tumor necrosis factor* (*TNF*)*-α* by human sebocytes (Q-PCR; **Figure 3b**). Moreover, as revealed by subsequent ELISA analyses, VDM11 treatment significantly suppressed LPS-induced release of IL-6, and led to only a minor, non-significant decrease in IL-8 secretion (24-hr treatments; **Figure 3c**). Concentrations of the other three cytokines were below (TNFα) or around (IL-1α and IL-1β) the detection limit of the respective assays (data not shown).

DISCUSSION

Skin dryness and the often accompanying overwhelming cutaneous inflammatory processes (e.g. in AD, etc.) can dramatically impair quality of life of many patients. On the other hand, appropriate moisturization and emollient treatment of the skin can alleviate symptoms, and, in some cases, they are even able to prevent the onset of AD (Hoppe et al. 2015; Oláh et al. 2017; Sawatzky et al. 2016; Sugiura et al. 2014). Although inappropriate epidermal ceramide production is thought to be the most important player in these processes, a growing body of evidence supports now the concept that dysregulation of SGs functions and the subsequent alterations in the SLP are also fundamental. Indeed, sebostasis, SG hypoplasia and reduced SLP (with pathologically reduced squalene and wax ester content) were shown to occur in AD, and an inverse correlation between the prevalence of acne and AD (the former characterized by increased, whereas the latter by decreased sebum production) was also observed (Shi et al. 2015). These findings, together with those ones demonstrating that sebaceous lipids are important regulators of the growth of cutaneous microbiota (Pappas 2009) collectively suggest that controlled, moderate “sebostimulation” (ideally without altering the physiological composition of the sebum) may be beneficial in diseases characterized by skin dryness.

The eCB signaling, a recently emerging, new regulator of cutaneous biology (Maccarrone et al. 2015), appears to be a very promising subject of study in this field. Indeed, we have previously shown that i) human sebocytes are able to produce the two major eCBs (AEA and 2-AG); ii) CB_2 receptor-coupled signaling contributes to the

maintenance of homeostatic SLP; and iii) direct AEA or 2-AG treatments dramatically increase lipogenesis (Dobrosi et al. 2008). On the other hand, (-)-cannabidiol, as well as several further non-psychotropic phytocannabinoids (e.g. (-)-Δ^9-tetrahydrocannabivarin) were shown to normalize arachidonic acid- and other mediators-induced excessive lipid synthesis, and exerted complex (combined lipostatic, anti-proliferative and anti-inflammatory) anti-acne effects, whereas others (namely (-)-cannabigerol and (-)-cannabigerovarin) were able to slightly, but significantly promote SLP (Oláh et al. 2016b; Oláh et al. 2014). Hence, in the current study we aimed to unveil yet hidden aspects of the ECS of human sebocytes.

Here we provide evidence that major members of the ECS (i.e. NAPE-PLD, DAGLα and –β, MAGL and FAAH) are expressed both *in vitro* in human sebocytes (**Supplementary Figure S1a-d**), and (with the sole exception of DAGLα, which exhibited dubious immunostaining pattern) also *in situ* in SGs of the human skin (**Figure 1**), which nicely confirms the available murine data of MAGL (Ma et al. 2011) and FAAH (Wohlman et al. 2016) expression in SGs. Moreover, besides the expression of the enzymes, we could also show that eCB transport is functionally active and pharmacologically inhibitable in human sebocytes (**Figure 2a**). This process is more likely to be involved in (re-)uptake/degradation rather than synthesis/release of the eCBs, since VDM11 (10 μM for 24 hours) significantly elevated AEA level in the samples, and tended to increase 2-AG concentration too. Interestingly, although this compound was proven to only negligibly alter FAAH-activity in sebocytes (**Supplementary Table S1**), we also observed a significant elevation in OEA levels, and a tendency towards increase in PEA concentrations (**Figure 2b-e**).

Next, we tested the effects on the viability, lipid synthesis and immune responses of human sebocytes. We found that non-cytotoxic concentrations of VDM11 and AM404 (**Supplementary Figures S3**, **S4a** and **S7**) induced a moderate, but significant increase in the SLP (**Figure 3a**; **Supplementary Figure S4b**), and that VDM11 interfered with the pro-inflammatory effects of LPS (**Figure 3b-c**). It is noteworthy that certain sebocyte-derived cytokines (e.g. IL-6) can induce differentiation of CD4+/CD45RA+ naïve T cells into T helper (Th)17 cells (Mattii et al. 2017), further supporting the concept that dysregulation of sebocyte biology can contribute not only to the development of acne, but also to other (partly) Th17-driven inflammatory dermatoses, e.g. AD or psoriasis. Thus, the suppression of IL-6 expression and release that we have demonstrated here (**Figure 3b-c**) is likely to be clinically relevant, and promises to be beneficial in such conditions.

Collectively, these findings strongly suggest that abrogation of sebocytes' eCB degradation, and the subsequent elevation of the "eCB-tone" promotes SLP. However, quite unexpectedly, by using the FAAH inhibitor URB597, we found that, albeit being successful in suppressing FAAH-activity of the sebocytes (**Supplementary Table S1**) it had no effect on SLP (**Supplementary Figure S5**). Explanation of this unexpected finding remains to be unveiled in future, targeted studies, but may lie in the fact that relatively low levels of FAAH activity (measured as the capability of cell membranes to hydrolyze radiolabeled AEA) was detected in SZ95 sebocytes. Further possible

interpretations about the possible underlying mechanisms are presented in the **Supplementary Discussion section 1**.

As discussed above, a moderate (i.e. not excessive, seborrheic/acnegenic) elevation of the physiological SLP would be highly desirable in the management of diseases accompanied by skin dryness (e.g. AD) (Pappas 2009; Shi et al. 2015). The fact that the lipogenic effect of VDM11 was far weaker than the ones usually seen upon direct AEA (**Figure 3a**), or other lipogenic (e.g. 2-AG, arachidonic acid and linoleic acid+testosterone) treatments (Dobrosi et al. 2008; Géczy et al. 2012; Oláh et al. 2016b; Oláh et al. 2014), together with the here reported anti-inflammatory effect (**Figure 3b-c**) indicates that VDM11 (and maybe other eCB transport inhibitors) may be beneficial in treating such diseases. Obviously, however, the exact impact of VDM11 treatment on the sebaceous lipidome must be thoroughly investigated in future specific studies in order to exclude the possibility of its potential "acnegenic" transformation.

It is noteworthy that anti-inflammatory effects of elevated eCB-tone are not unprecedented either in murine or in human skin. Indeed, since the groundbreaking work of Karsak and her co-workers, many other studies showed that the homeostatic ECS is one of the master regulators of cutaneous immune responses, keeping under control local allergic and inflammatory processes (Karsak et al. 2007; Oláh et al. 2016a).

Moreover, the fact that VDM11 could significantly increase OEA, and tended to elevate PEA levels (**Figure 2d-e**) highlights the possibility that SGs may contribute to the cutaneous PEA and OEA metabolism and supply of their local micromilieu with these pleiotropic anti-inflammatory molecules (Facci et al. 1995; Impellizzeri et al. 2015; Pontis et al. 2016; Yang et al. 2016). Based on these data, there is a possibility that SG hypoplasia and hypofunction observed in AD (Shi et al. 2015) may be accompanied not only by reduced sebum production, but also by impaired PEA (and/or OEA) supply, which might contribute to the development and worsening of atopic inflammation. Considering that a PEA containing cream was recently shown to efficiently alleviate symptoms of AD patients ("ATOPA study") (Eberlein et al. 2008), clinical studies are urgently invited to explore the possible role of SG hypoplasia-derived putative PEA/OEA-deficiency in the development of AD symptoms. Further speculations about the possible role and targets of PEA and OEA are mentioned in the **Supplementary Discussion section 2**.

Collectively, our findings demonstrate that i) human sebocytes express the most important enzymes involved in eCB and eCB-like mediator metabolism; ii) are involved in the cutaneous metabolism of PEA and OEA; and that VDM11 iii) increases or tends to increase the levels of eCBs and related acylethanolamides; (iv) induces a moderate increase in SLP; and (v) remarkable anti-inflammatory actions in human sebocytes (summarized in **Supplementary Figure S8**). Thus, human SGs may be important in regulating the supply of key eCBs and related acylethanolamides to their tissue microenvironment, and targeting the ECS holds out promise to control cutaneous inflammation. All in all, our data should encourage the exploitation of selected, SG-

targeting ECS-modulators in appropriate clinical trials, to alleviate symptoms of cutaneous diseases (e.g. AD) characterized by skin dryness and inflammation.

MATERIALS AND METHODS

Detailed description of the applied materials and methods can be found in the **Supplementary Methods and Methods section**. Briefly, lipid synthesis was investigated by fluorescent Nile Red staining, viability and cell death was assessed by MTT-assay and combined fluorescent $DilC_1(5)$-SYTOX Green labeling, respectively. Gene expression was studied by Q-PCR (mRNA level), ELISA, Western blot and immunohistochemistry (protein level). The uptake of AEA into cells was determined by measuring the cellular uptake of radiolabeled AEA as described before (Nicolussi et al. 2014b; Nicolussi et al. 2014a), whereas levels of the eCBs were quantified by isotope dilution-liquid chromatography coupled to single quadrupole mass spectrometric (LC-MS) analysis (Marsicano et al. 2002), and FAAH-activity was determined according to our previously established and optimized protocol (Ortar et al., 2003). Data were analyzed by Origin Pro Plus 6.0 software (Microcal, Northampton, MA, USA), using Student's two tailed, unpaired *t*-test and $P<0.05$ values were regarded as significant differences. Graphs were plotted by using Origin Pro Plus 6.0 software (Microcal). Primary human material was collected after obtaining written informed consent, adhering to Helsinki Declaration, and after obtaining Institutional Research Ethics Committee's and Government Office for Hajdú-Bihar County's permission (document IDs: IX-R-052/01396-2/2012, IF-12817/2015, IF-1647/2016, IF-778-5/2017).

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CONFLICT OF INTEREST

CCZ owns an international patent on the SZ95 sebaceous gland cell line (WO2000046353).

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FIGURE LEGENDS

Figure 1 *Major enzymes of the endocannabinoid metabolism are expressed in human sebaceous glands in situ*

Immunohistochemistry of human skin sections was performed as described in the Supplementary Methods section. Specific immunopositivity was visualized by 3,3'-diaminobenzidine (DAB; brown color), whereas nuclei were counterstained by hematoxylin (blue color). Original magnifications: 100x (left column), 400x (middle and right columns); scale bars: 200 µm (left column) and 50 µm (middle and right columns). Arrows indicate sweat glands (endogenous positive control for DAGLα), whereas arrowheads mark sebaceous glands on the same image. Negative controls (right column) were obtained by omitting the primary antibody in all cases. **DAGL:** diacylglycerol lipase; **FAAH:** fatty acid amide hydrolase; **MAGL:** monoacylglycerol lipase; **NAPE-PLD:** *N*-acyl phosphatidylethanolamine-specific phospholipase D.

Figure 2 *Effects of the inhibitors of the putative endocannabinoid membrane transporter in human sebocytes*

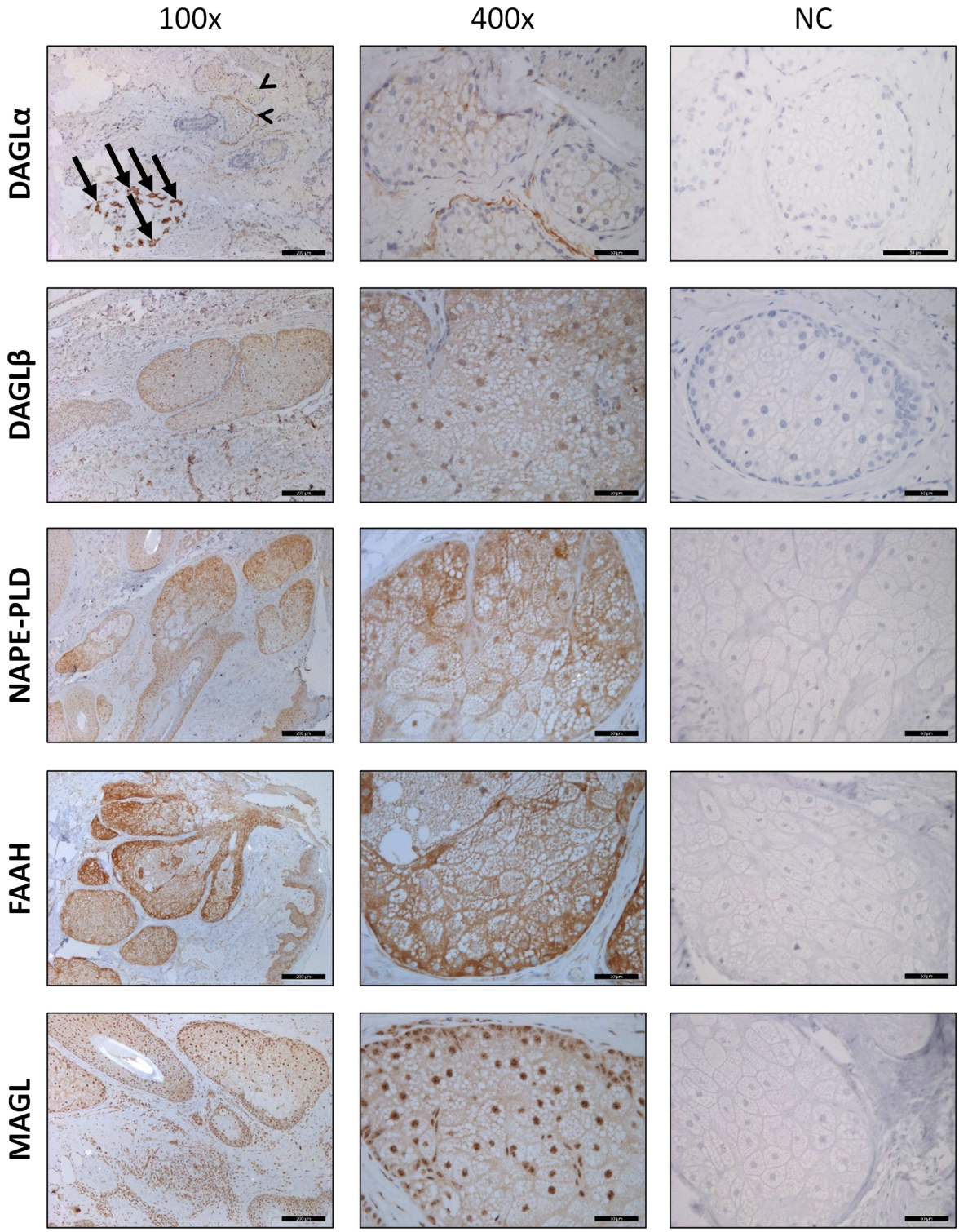
(**a**) AEA transport measurement. Cells were treated with the reference AEA uptake inhibitor UCM707 or vehicle for 15 min, and intra- and extracellular amounts of radiolabeled AEA or $EtNH_2$ were detected as described in the Supplementary Methods section. Results are expressed in the percentage of the vehicle control (100%, solid line) as mean±SEM of three independent experiments, each run in triplicate. (**b-e**) AEA, 2-AG, PEA and OEA determination of the samples (i.e. cells and their supernatants together) was performed as described in the Supplementary Methods section. Results

are expressed as mean±SEM of 3-4 independent cultures. *, ** and *** mark significant (P<0.05, 0.01 or 0.001, respectively) differences compared to the vehicle control. n.s.: not significant difference. **2-AG:** 2-arachidonoylglycerol; **AEA:** N-arachidonoylethanolamine (anandamide); **EMT:** (putative) endocannabinoid membrane transporter; **OEA:** oleoylethanolamide; **PEA:** palmitoylethanolamide; **UCM707** and **VDM11:** EMT-inhibitors.

Figure 3 *Non-cytotoxic concentrations of VDM11 moderately, but significantly increase sebaceous lipid synthesis, and exert remarkable anti-inflammatory effects*

(**a**) 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. One additional experiment yielded similar results. ** and *** mark significant (P<0.01 and 0.001, respectively) differences compared to the vehicle control. ###P<0.001. n.s.: not significant difference compared to the vehicle control. (**b**) Q-PCR. *IL-1α*, *IL-1β*, *IL-6*, *IL-8* and *TNF-α* mRNA expressions were determined following 3-hr LPS-treatment with or without VDM11. 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 three determinations. One additional experiment yielded similar results. ** and ***P<0.01 and 0.001, respectively, as indicated. (**c**) ELISA. IL-6 and IL-8 content of the sebocyte supernatants was determined following 24-hr LPS-treatment with or without VDM11. Data are expressed as mean±SD of three determinations. Two additional experiments yielded similar results. ***P<0.001, as indicated. n.s.: not significant difference. **AEA:** N-arachidonoylethanolamine

(anandamide); **EMT:** endocannabinoid membrane transporter; **IL:** interleukin; **LPS:** lipopolysaccharide; **TNF:** tumor necrosis factor; **VDM11:** EMT-inhibitor.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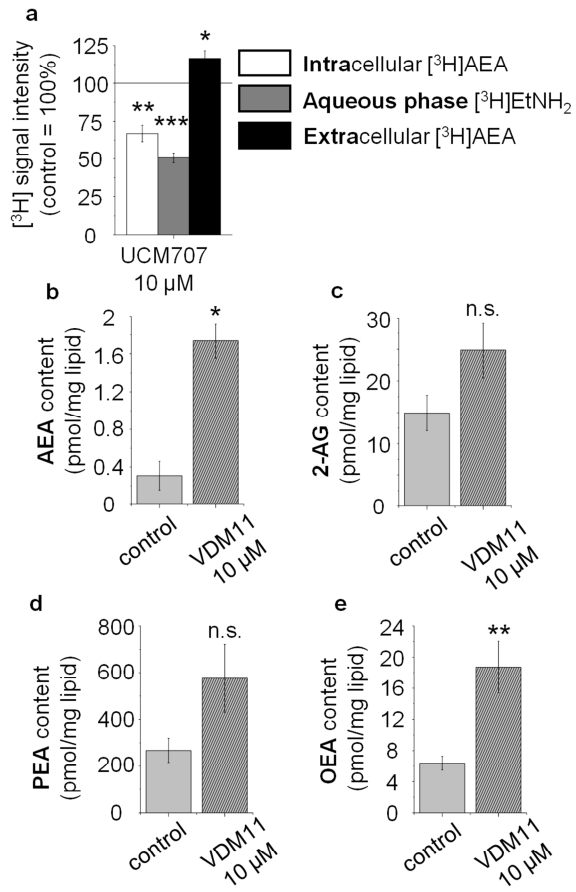
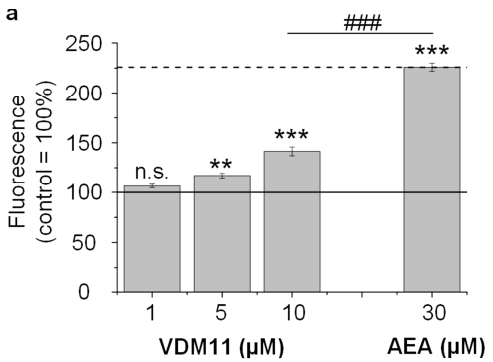

a
[3H] signal intensity (control = 100%)
125
100
75
50
25
0
**
***
*
Intracellular [3H]AEA
Aqueous phase [3H]EtNH2
Extracellular [3H]AEA
UCM707
10 μM
b
AEA content (pmol/mg lipid)
2
1.6
1.2
0.8
0.4
0
*
control
VDM11
10 μM
c
2-AG content (pmol/mg lipid)
30
20
10
0
n.s.
control
VDM11
10 μM
d
PEA content (pmol/mg lipid)
800
600
400
200
0
n.s.
control
VDM11
10 μM
e
OEA content (pmol/mg lipid)
24
20
16
12
8
4
0
**
control
VDM11
10 μM


a
###
***
250
200
150
100
50
0
Fluorescence
(control = 100%)
n.s.
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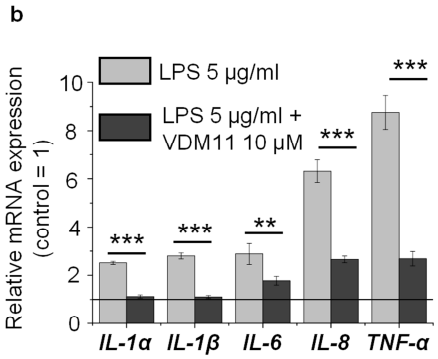
1
5
10
30
VDM11 (μM)
AEA (μM)


b
LPS 5 μg/ml
LPS 5 μg/ml +
VDM11 10 μM
***
***
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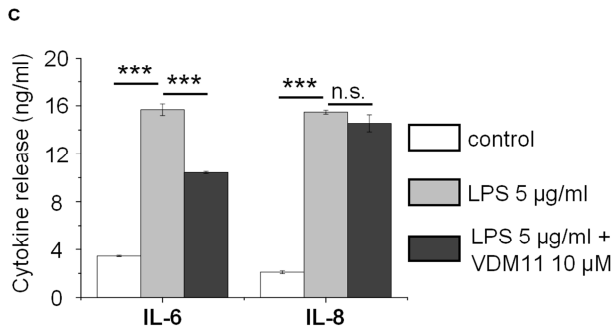
**
***
10
8
6
4
2
0
Relative mRNA expression
(control = 1)
IL-1α
IL-1β
IL-6
IL-8
TNF-α


c
***
***
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n.s.
20
16
12
8
4
0
Cytokine release (ng/ml)
IL-6
IL-8
control
LPS 5 μg/ml
LPS 5 μg/ml +
VDM11 10 μM