Endocannabinoid Tone Regulates Human Sebocyte Biology

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We have previously shown that endocannabinoids (eCBs) (e.g., anandamide) are involved in the maintenance of homeostatic sebaceous lipid production in human sebaceous glands and that eCB treatment dramatically increases sebaceous lipid production. 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, which has been hypothesized to facilitate the cellular uptake and subsequent degradation of eCBs. We found that the major eCB synthesizing (*N*-acyl phosphatidylethanolamine-specific phospholipase D, and diacylglycerol lipase-α and -β) and degrading (fatty acid amide hydrolase, monoacylglycerol lipase) enzymes are expressed in SZ95 sebocytes and also in sebaceous glands (except for diacylglycerol lipase-α, the staining of which was dubious in histological preparations). eCB uptake-inhibition with VDM11 induced a moderate increase in sebaceous lipid production and also elevated the levels of various eCBs and related acylethanolamides. Finally, we found that VDM11 was able to interfere with the proinflammatory action of the TLR4 activator lipopolysaccharide. Collectively, our data suggest that inhibition of eCB uptake exerts anti-inflammatory actions and elevates both sebaceous lipid production and eCB levels; thus, these inhibitors might be beneficial in cutaneous inflammatory conditions accompanied by dry skin.

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INTRODUCTION

Sebaceous glands (SGs) are important players and regulators of human skin homeostasis. In addition to their obvious function, that is, production of lipid-rich sebum, by which they contribute 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 (Dajnoki et al., 2017; Lupi, 2008; Porter, 2001; Tóth et al., 2011; Zouboulis et al., 2008, 2014). Their clinical significance is also remarkably high, because 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., 2008, 2014). On the other hand, lack of sufficient sebum production in adulthood may contribute to dry skin syndrome, xerosis, or even skin aging 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, the unique composition of sebum is thought to play an important role in regulating the growth of the cutaneous microbiota by restricting unwanted microbes and promoting preferred ones, thus making homeostatic SG functions important orchestrators of skin-microbiota crosstalk (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 the (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, because primary sebocytes cannot be kept in culture for more than a few passages, and there are no adequate animal model systems for assessing the whole complexity of human SG biology (Tóth et al., 2011; Zouboulis et al., 2008, 2014). Therefore, in this study, we used the human immortalized SZ95 sebocyte cell line (Zouboulis et al. 1999), which is a widely accepted model system for studying human SG functions in vitro (Tóth et al., 2011; Zouboulis et al., 2008, 2014).

A growing body of evidence suggests that SGs are not only "innocent" targets of the complex cutaneous neuroendocrine

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Abbreviations: 2-AG, 2-arachidonoylglycerol; AD, atopic dermatitis; AEA, arachidonoylethanolamide; DAGL, diacylglycerol lipase; eCB, endocannabinoid; ECS, endocannabinoid system; EMT, endocannabinoid membrane transporter; FAAH, fatty acid amide hydrolase; LPS, lipopolysaccharide; MAGL, monoacylglycerol lipase; NAPE-PLD, N-acyl phosphatidylethanolamine-specific phospholipase D; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; SG, sebaceous gland; SLP, sebaceous lipid production

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system (Slominski, 2005; Slominski and Wortsman, 2000), but also act as 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., 2008, 2014). 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 [eCBs] such as arachidonoylethanolamide [AEA], also known as anandamide; 2-arachidonoylglycerol [2-AG]); and receptors (e.g., CB_1 and CB_2, etc.) and 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, such as 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). We have previously shown that human SGs are capable of producing the major eCBs (namely AEA and 2-AG), and we could also show, in line with the early data of Ständer et al. (2005) that 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, because 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 evidence, to our knowledge previously unreported, that human SGs have 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 this highly focused study, we aimed to explore the expression of the major members of the ECS in vitro in human sebocytes and in situ in human skin, and we also wanted to investigate whether pharmacological modulation of eCB homeostasis was indeed able to regulate SLP.

RESULTS

Major enzymes of eCB metabolism are expressed in cultured human sebocytes and 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 mentioned enzymes were expressed in human sebocytes both at the mRNA (quantitative PCR) and protein (Western blot) levels (see Supplementary Figure S1a–d online). To further confirm these results, we also investigated their expression in human skin samples (appropriately labeled positive controls are shown in Supplementary Figure S2 online). With the sole exception of DAGLα, which exhibited questionable expression compared with the endogenous positive control sweat glands (Czifra et al., 2012), our findings nicely confirmed our in vitro data about the expression of these enzymes in human SGs in situ (Figure 1).

Sebocytes exhibit a pharmacologically inhibitable eCB uptake process

Next, we assessed whether 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 minutes, 10 μmol/L) 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. UCM707 reduced the intracellular [^{3}H]AEA signal, which was accompanied by a significant increase in extracellular [^{3}H]AEA levels compared with 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 was reduced (Figure 2a). Theoretically, a decrease of [^{3}H]AEA uptake and [^{3}H]ethanolamine levels could be explained not only by the inhibition of the eCB transport process, but also by nonspecific 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 (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 compared with the reference FAAH inhibitor URB597 (Mor et al., 2004). We found that SZ95 sebocytes exhibit very low constitutive FAAH activity (2.32 ± 0.25 pmol/minute/mg protein, $n = 10$), and that, unlike URB597, neither UCM707 nor VDM11 exerted substantial FAAH inhibition (half maximal inhibitory concentration values were above 25 μmol/L for both EMT inhibitors, whereas the half maximal inhibitory concentration of URB597 was below 100 nmol/L) (see Supplementary Table S1 online). Taken together, these findings suggest that in human sebocytes, eCB uptake can be inhibited by VDM11, most likely in a FAAH-independent manner.

The 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 liquid chromatography-mass

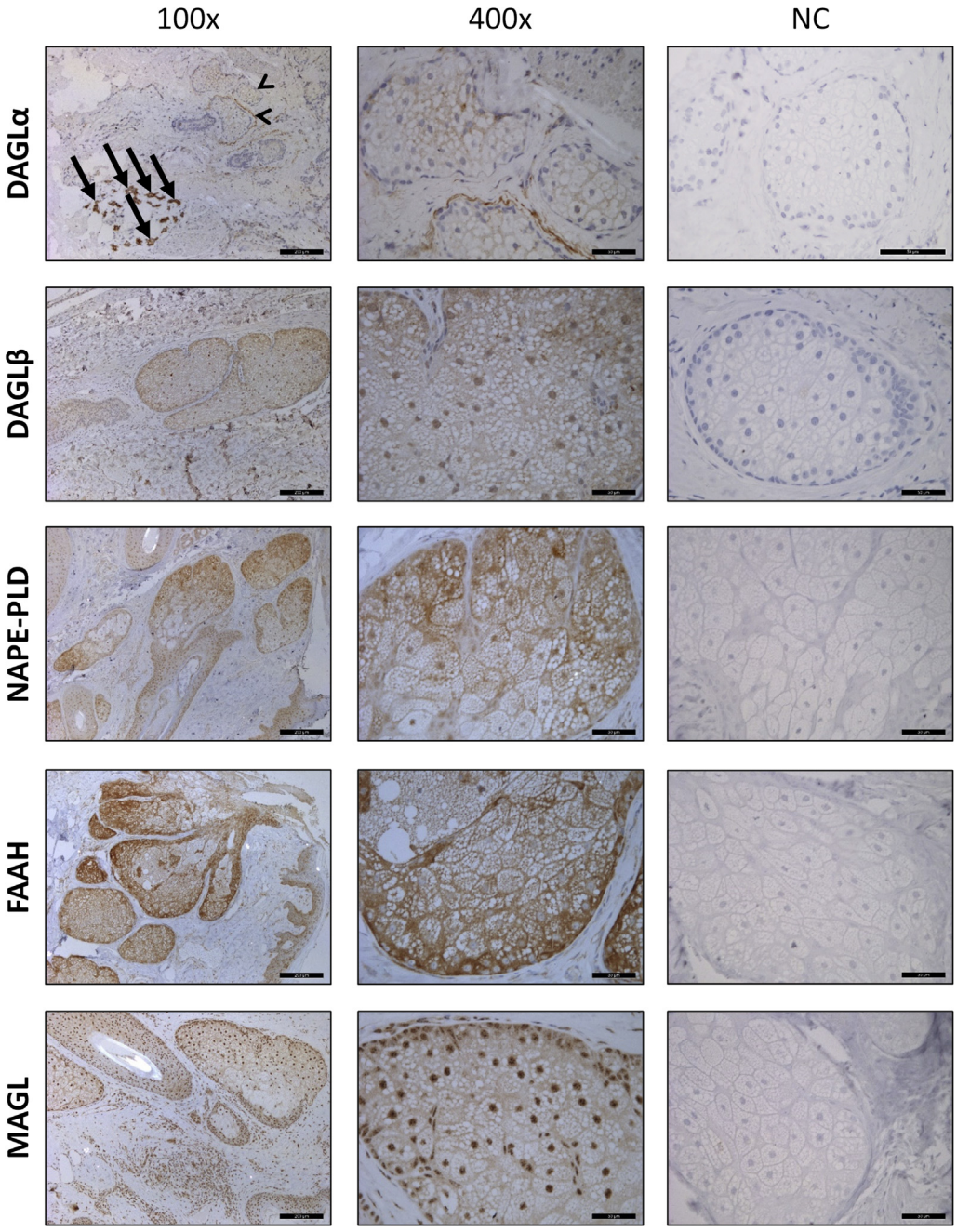

Figure 1. Major enzymes of the endocannabinoid metabolism are expressed in human sebaceous glands in situ. Immunohistochemistry of human skin sections was performed as described in the Supplementary Materials. Specific immunopositivity was visualized by 3,3′-diaminobenzidine (brown color), and nuclei were counterstained with hematoxylin (blue color). Original magnifications = ×100 in the left column and ×400 in the middle and right columns. Scale bars = 200 μm in the left column and 50 μm in the middle and right columns. Arrows indicate sweat glands (endogenous positive control for DAGLα), and 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, NC, negative control.

spectrometry. 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, PEA and OEA. We found that treatment by VDM11 tended to increase PEA and significantly elevated OEA concentrations (Figure 2d and 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 suggest 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 sebocytes results in dramatically increased SLP (Dobrosi et al., 2008; Oláh et al., 2014, 2016b). Thus, next we wanted to assess how treatment with eCB uptake inhibitors influences the functions of SGs. Using noncytotoxic concentrations (determined by MTT assay; see Supplementary Figure S3 online) 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 SLP after 48-hour treatments (Figure 3a). Repetition of the experiment with AM404

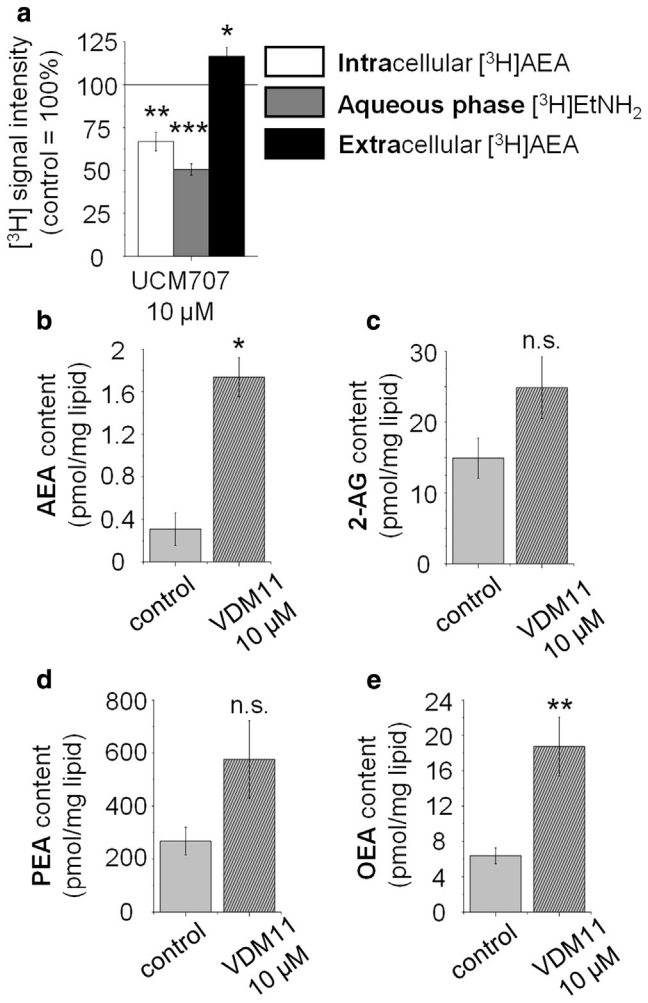

Figure 2. Effects of the inhibitors of the putative endocannabinoid membrane transporter in human sebocytes. (**a**) AEA transport measurement. Cells were treated with the reference AEA uptake inhibitor UCM707 or vehicle for 15 minutes, and intra- and extracellular amounts of radiolabeled AEA or $EtNH_2$ were detected as described in the Supplementary Materials. Results are expressed as the percentage of vehicle control (100%, solid line) as mean ± standard error of the mean of three independent experiments, each run in triplicate. (**b**–**e**) AEA, 2-AG, PEA, and OEA determinations of the samples (i.e., cells and their supernatants together) were performed as described in the Supplementary Materials. Results are expressed as mean ± standard error of the mean of 3 or 4 independent cultures. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ mark significant differences compared with the vehicle control. 2-AG, 2-arachidonoylglycerol; AEA, N-arachidonoylethanolamine (i.e., anandamide); $EtNH_2$, ethanolamine; EMT, (putative) endocannabinoid membrane transporter; M, mol/L; n.s., not significant; OEA, oleoylethanolamide; PEA, palmitoylethanolamide.

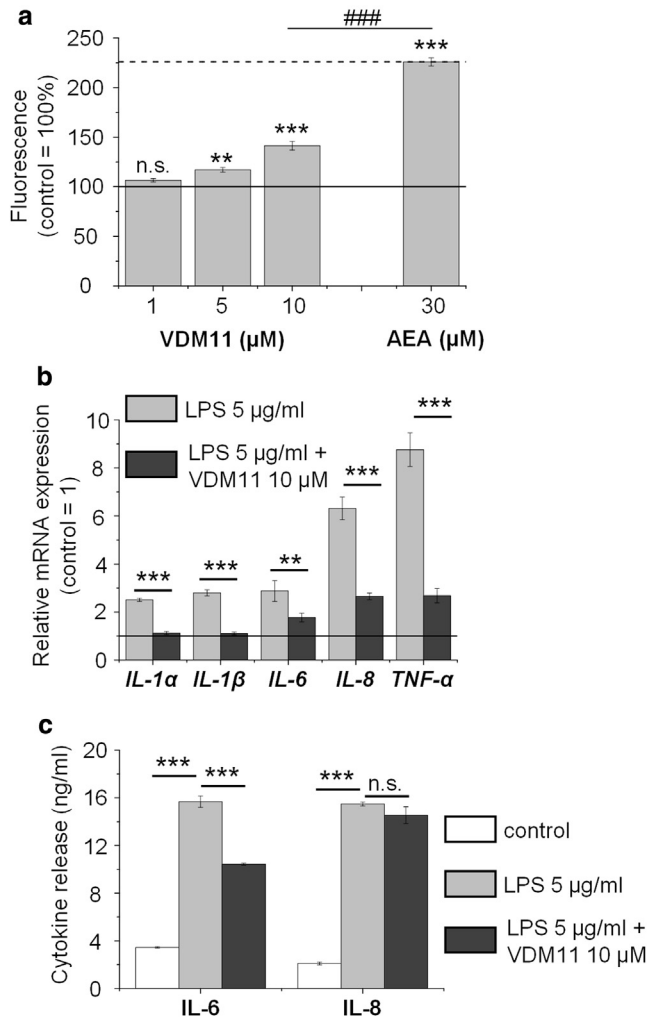

Figure 3. Noncytotoxic concentrations of VDM11 moderately but significantly increase sebaceous lipid synthesis and exert remarkable anti-inflammatory effects. (**a**) Sebaceous lipid production of SZ95 sebocytes was assessed by Nile Red staining after 48-hour treatments. Results are expressed as the percentage of the vehicle control (100%, solid line) as mean ± standard error of the mean of four independent determinations. One additional experiment yielded similar results. $^{**}P < 0.01$ and $^{***}P < 0.01$ mark significant differences compared with the vehicle control. $^{###}P < 0.001$. (**b**) Quantitative PCR. *IL-1α, IL-1β, IL-6, IL-8* and *TNF-α* mRNA expressions were determined after 3-hour LPS treatment with or without VDM11. 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 ± standard deviation of three determinations. One additional experiment yielded similar results. $^{**}P < 0.01$ and $^{***}P < 0.001$, as indicated. (**c**) ELISA. IL-6 and IL-8 content of the sebocyte supernatants was determined after 24-hour LPS treatment with or without VDM11. Data are expressed as mean ± standard deviation of three determinations. Two additional experiments yielded similar results. $^{***}P < 0.001$, as indicated. AEA, *N*-arachidonoylethanolamine (i.e., anandamide); LPS, lipopolysaccharide; M, mol/L; n.s., not significant compared with the vehicle control.

(another well-known AEA uptake inhibitor) (Beltramo et al., 1997; Nicolussi and Gertsch, 2015) yielded very similar results (see Supplementary Figure S4a and b online). 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, several lines of evidence show 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 of the FAAH activity of sebocytes 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 μmol/L without the risk of having substantial impact on FAAH activity (see Supplementary Table S1), we also intended to investigate biological effects of the selective reference FAAH inhibitor URB597 (Mor et al., 2004). We found that

administration of URB597 led to a different cell physiology outcome than application of VDM11 and AM404. Indeed, our data showed that noncytotoxic concentrations (MTT assay, see Supplementary Figure S5a online) of URB597 did not influence SLP (48-hour treatments, see Supplementary Figure S5b) compared with 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. 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 assessed how co-administration of AEA and VDM11 affect SLP. We found that VDM11 was unable to further promote the AEA-induced, already elevated SLP (see Supplementary Figure S6 online) of human sebocytes, suggesting that the pro-lipogenic eCB signaling activated by 30 μmol/L AEA is exhaustive and has no further "reserve capacity."

Up to 10 μmol/L, 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., 2008, 2014). Because VDM11 was shown to moderately promote SLP (Figure 3a, and see Supplementary Figure S4b), we also wanted to know if it induced early apoptotic processes. We found that, although the most effective lipogenic concentration of VDM11 tended to decrease mitochondrial membrane potential in course of 48-hour treatments (see Supplementary Figure S7 online), this did not reach the level of significance, suggesting that within the studied timeframe, it may indeed be devoid of obvious pro-apoptotic effects.

VDM11 suppresses lipopolysaccharide (LPS)-induced proinflammatory 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 results suggest that administration of VDM11 (and probably other EMT inhibitors too) may have 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 LPS (5 μg/ml, 3-hour treatments) (Oláh et al., 2016b) to induce a proinflammatory response. Co-administration of VDM11 (10 μmol/L) efficiently suppressed LPS-induced expression of *IL-1α, IL-1β, IL-6, IL-8,* and *TNF-α* by human sebocytes (quantitative PCR) (Figure 3b). Moreover, as shown by subsequent ELISA analyses, VDM11 treatment significantly suppressed LPS-induced release of IL-6 and led to only a minor, nonsignificant decrease in IL-8 secretion (24-hour treatments) (Figure 3c). Concentrations of the other three cytokines were below (TNF-α) 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 now supports the concept that dysregulation of SG 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, and the latter by decreased, sebum production) was also observed (Shi et al., 2015). These findings, together with those showing 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.

eCB signaling, a recently emerging regulator of cutaneous biology (Maccarrone et al., 2015), appears to be a very promising subject of study in this field. 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 and several further nonpsychotropic phytocannabinoids (e.g., (−)-Δ^9-tetrahydrocannabivarin) were shown to normalize arachidonic acid- and other mediator-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., 2014, 2016b). Hence, in this study we aimed to unveil 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 (see Supplementary Figure S1a–d) and (with the sole exception of DAGLα, which exhibited a 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, because VDM11 (10 μmol/L for 24 hours) significantly

elevated AEA levels in the samples and tended to increase 2-AG concentration as well. Although this compound was proven to only negligibly alter FAAH activity in sebocytes (see Supplementary Table S1), we also observed a significant elevation in OEA levels and a tendency toward 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 noncytotoxic concentrations of VDM11 and AM404 (see Supplementary Figures S3, S4a, and S7) induced a moderate but significant increase in SLP (Figure 3a, and see Supplementary Figure S4b) and that VDM11 interfered with the proinflammatory effects of LPS (Figure 3b and c). Certain sebocyte-derived cytokines (e.g., IL-6) can induce differentiation of $CD4^+/CD45RA^+$ naïve T cells into T helper type 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) T helper type 17-driven inflammatory dermatoses, such as AD or psoriasis. Thus, the suppression of IL-6 expression and release that we have shown here (Figure 3b and c) is likely to be clinically relevant and promises to be beneficial in such conditions.

Collectively, these findings strongly suggest that abrogation of eCB degradation of sebocytes, and the subsequent elevation of the "eCB-tone," promotes SLP. However, quite unexpectedly, by using the FAAH inhibitor URB597, we found that, although it was successful in suppressing the FAAH activity of the sebocytes (see Supplementary Table S1), it had no effect on SLP (Supplementary Figure S5). An explanation of this unexpected finding remains to be uncovered 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 Materials online.

As discussed, a moderate (i.e., not excessive, seborrheic/acnegenic) elevation of 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 those 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., 2014, 2016b), together with our reported anti-inflammatory effect (Figure 3b and 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 to exclude the possibility of its potential acnegenic transformation.

Anti-inflammatory effects of elevated eCB tone are not unprecedented in either murine or human skin. Since the groundbreaking work of Karsak et al. (2007), many other studies have shown that the homeostatic ECS is one of the master regulators of cutaneous immune responses, keeping under control local allergic and inflammatory processes (Oláh et al., 2016a).

Moreover, the fact that VDM11 could significantly increase OEA and tended to elevate PEA levels (Figure 2d and 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. Because 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 Materials.

Our findings show that (i) human sebocytes express the most important enzymes involved in eCB and eCB-like mediator metabolism; (ii) human sebocytes are involved in the cutaneous metabolism of PEA and OEA; (iii) VDM11 increases or tends to increase the levels of eCBs and related acylethanolamides; (iv) VDM11 induces a moderate increase in SLP; and (v) VDM11 induces remarkable anti-inflammatory actions in human sebocytes (see Supplementary Figure S8 online). 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 descriptions of the applied materials and methods can be found in the Supplementary Materials. Briefly, lipid synthesis was investigated by fluorescent Nile Red staining, and viability and cell death were assessed by MTT assay and combined fluorescent $DiIC_1(5)$-SYTOX Green labeling, respectively. Gene expression was studied by quantitative 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., 2014a, 2014b), whereas levels of the eCBs were quantified by isotope dilution-liquid chromatography coupled with single quadrupole mass spectrometric 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) using the Student two-tailed, unpaired *t* test, and *P*-values less than 0.05 were regarded as significant differences. Graphs were plotted by using Origin Pro Plus 6.0 software. Primary human material was collected after obtaining written informed consent, adhering to the Helsinki Declaration, and after obtaining permission from the Institutional Research Ethics Committee and Government Office for Hajdú-Bihar County

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CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.02.022.

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SUPPLEMENTARY MATERIAL

Endocannabinoid tone regulates human sebocyte biology

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SUPPLEMENTARY METHODS

Materials

VDM11 ((5Z,8Z,11Z,14Z)-N-(4-Hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide) and AM404 (N-(4-Hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide) together with AEA were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). URB597 (Cyclohexylcarbamic acid 3'-(Aminocarbonyl)-[1,1'-biphenyl]-3-yl ester) and UCM707 ((5Z,8Z,11Z,14Z)-N-(3-Furanylmethyl)-5,8,11,14-eicosatetraenamide) were obtained from were obtained from Tocris Bioscience (Bristol, UK) or Cayman Chemical Company (Ann Arbor, MI, USA and Tallinn, Estonia; in case of AEA uptake assay); [ethanolamine-1-^{3}H]-AEA (60 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA); whereas manufacturer of γ-irradiated lipopolysaccharide from *Escherichia coli* 026:B6 (LPS) was Sigma-Aldrich (St. Louis, MO, USA), respectively. LPS was dissolved in filtered distilled water. The solvent of all other compounds was absolute ethanol (Sigma-Aldrich) except for the [^{3}H]-AEA uptake assay where UCM707 was dissolved in DMSO. Control cultures were always treated with appropriate amount of vehicles.

Cell culturing

Human immortalized SZ95 sebocytes, originated from human facial sebaceous glands (Zouboulis et al., 1999; Tóth et al., 2011; Zouboulis et al., 2014), were cultured in Sebomed® Basal Medium (Biochrom, Berlin, Germany) supplemented with 10 (V/V)% fetal 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 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 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

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 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 (Green and Reed, 1998; Susin et al., 1998). Therefore, to assess the process, mitochondrial membrane potential of SZ95 sebocytes was determined using 1,1′,3,3,3′,3′-hexamethylindodicarbo-cyanine iodide containing MitoProbe™ $DilC_1(5)$ 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 48 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 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).

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 48 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 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.

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.

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 IDs: Hs00174092_m1 for *interleukin* [*IL*]-*1α*,

Hs00174097_m1 for *IL-1β,* Hs00985639_m1 for *IL-6*, Hs00174103_m1 for *IL-8*, Hs00174128_m1 for *tumor necrosis factor* [*TNF*]-*α;* Hs00419593_m1 for *NAPE-PLD;* Hs00391374_m1 for *DAGLα;* Hs00373700_m1 for *DAGLβ,* Hs00155015_m1 for *FAAH* and Hs00200752_m1 for *MAGL*) and the TaqMan universal PCR master mix protocol (Applied Biosystems). As internal control, transcripts of *18S RNA* or peptidylprolyl isomerase A (PPIA) were determined (assay IDs: Hs03928905_g1 and Hs99999905_m1, respectively). The amount of the transcripts was normalized to those of the housekeeping gene using the ΔCT method. Finally, when indicated, the results were further normalized to the expression of the vehicle control (ΔΔCT method).

Determination of cytokine release (ELISA)

500,000 cells were seeded in Petri-dishes (d=15 mm) in 1.5 ml culture medium. On the next day, cells were treated as indicated. Supernatants were collected, and the released amounts of IL-1α, IL-1β, IL-6, IL-8, and TNFα were determined according to the manufacturers' (IL-1β, IL-6, IL-8, and TNFα: BD Pharmingen, Franklin Lakes, NJ, USA; IL-1α: R&D Systems, Inc., Minneapolis, MN, USA) protocols. In case of IL-6 and IL-8, supernatants were diluted (1:50), whereas for the other three cytokines, non-diluted supernatants were used.

Western blotting

Cells were harvested in lysis buffer (20 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1 mM 4-(2-aminoethyl) benzensulphonyl fluoride, protease inhibitor cocktail diluted 1:100, (all from Sigma-Aldrich) and the protein content was measured by a modified BCA protein assay (Pierce, Rockford, IL, USA). The samples were then subjected to sodium dodecyl

sulphate-polyacrylamide gel electrophoresis. 10% Mini Protean TGX gels (Bio-Rad, Hercules, CA, USA) were loaded with equal (40 μg) amount of protein per lane. Samples were then transferred to nitrocellulose membranes, by using Trans-Blot® Turbo™ Nitrocellulose Transfer Packs and Trans-Blot Turbo™ System (both from Bio-Rad), and then probed with mouse-anti-human FAAH, rabbit-anti-human NAPE-PLD, MAGL and DAGLα (all from Abcam, Camebridge, UK), or goat-anti-human DAGLβ (Santa Cruz Inc., Heidelberg, Germany) specific primary antibodies (all overnight at 4°C in 1:200 in 5% milk containing PBS). As secondary antibody, horseradish peroxidase-conjugated rabbit IgG Fc segment-specific antibodies (developed in goat, 1:1000 in 5% milk containing PBS, Bio-Rad) were used, and the immunoreactive bands were visualized by a SuperSignal® West Pico Chemiluminescent Substrate enhanced chemiluminescence kit (Pierce) using a KODAK Gel Logic 1500 Imaging System (Eastman Kodak Company, Kodak, Tokyo, Japan).

Immunohistochemistry

The immunohistochemical investigation of NAPE-PLD, FAAH, MAGL (Novus Biologicals, Littleton, USA) and DAGLβ (Bioss Inc., Massachusetts, USA) was performed on 3 formalin fixed paraffin embedded, skin samples rich in sebaceous glands, all diagnosed as trichilemmal cyst. The expression pattern of DAGLα (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in sebaceous gland was examined on 3 frozen skin samples obtained from vertex.

Serial 4 μm thick sections were cut from paraffin blocks and frozen tissues as well. Frozen sections were then fixed in pre-cooled acetone for 10 min, whereas heat-induced

antigen retrieval was performed on formalin fixed samples. FAAH, MAGL, and DAGLβ epitopes were retrieved in 11 mM citrate buffer (pH 6) applied in pressure cooker for 2 min on full pressure. NAPE-PLD was retrieved in EnVision FLEX Target Retrieval Solution High pH (DAKO, Glostrup, Denmark) in water bath for 30 min at 95 °C. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 minutes in case of formalin fixed and frozen sections as well. After blocking, tissue sections were incubated at room temperature with primary antibodies diluted in 1% Bovine Serum Albumin (Sigma-Aldrich, St. Louis, MO, USA): NAPE-PLD (mouse, clone: 5F7, 1:75, 60 min), FAAH (rabbit polyclonal, 1:20, 60 min), MAGL (mouse, clone: 2B11, 1:100, 60 min), DAGLα (rabbit polyclonal, 1:100, 30 min), DAGLβ (rabbit polyclonal, 1:50, 30 min). Sections were then incubated with the EnVision FLEX Labeled polymer-HRP anti-rabbit and anti-mouse System (DAKO, Glostrup, Denmark) at room temperature for 30 min with 3,3'-diaminobenzidine (DAB) visualization techniques. Cell nuclei were counterstained with hematoxylin and tissue sections were finally mounted in permanent mounting medium (Histolab, Göteborg, Sweden).

Tissues with known antigen expression pattern were used in every staining run as positive controls (**Supplementary Figure S2**). Renal tubules served as positive controls for NAPE-PLD (expected positivity: membranes of the proximal tubules according to The Human Protein Atlas http://www.proteinatlas.org/ENSG00000161048-NAPEPLD/tissue) and MAGL (expected positivity: nuclei in all tubules; Labar et al., 2010) detection. Testis was used to validate FAAH expression (expected positivity: cytoplasmic staining in Leydig cells and in testicular tubules; Scotchie et al., 2015), and cerebellum was applied as a positive control for DAGLα (expected positivity: dendritic

tree of Purkinje cells; Bisogno et al., 2003) and DAGLβ (expected positivity: synaptic areas; Bisogno et al., 2003). Negative controls were obtained by omitting the primary antibody in all cases.

[^{3}H]-AEA uptake assay in SZ95 sebocytes

The cellular uptake of AEA in SZ95 sebocytes was determined using an established multi-phase assay protocol previously described for different cell types (Nicolussi et al., 2014a; Nicolussi et al., 2014b, Rau et al. 2014). Briefly, 1 Mio. SZ95 sebocytes per sample were incubated with either the vehicle control DMSO (5 µL) or the reference AEA uptake inhibitor UCM707 (5 µL, 10 µM final) in a volume of 500 µL RPMI, 37°C in AquaSil™ silanized screw-cap Eppendorf tubes. After 15 min of incubation at 37°C a mixture of [*ethanolamine*-1-^{3}H]AEA (0.5 nM) and unlabelled AEA (final 100 nM) in 5 µL EtOH was added for 15 min. The uptake process was stopped by putting the samples on ice following rapid centrifugation at 800 x g for 5 min at 4°C. The supernatant was collected separately and the cell pellet was washed and resuspended using 500 µL ice-cold PBS containing 1 % BSA (fatty acid-free). The washing solution was collected and pooled with the organic phase extracted from the primary supernatant after phase separation (1:1 $CHCl_3$:MeOH, ice cold, 1 mL, 10'000 x g) resulting in the extracellular phase. The aqueous phase was collected separately. The cell pellet was resuspended in 250 µL PBS. All phases were transferred into scintillation tubes and merged with Ultima Gold scintillation cocktail following liquid scintillation counting (LCS) using a Packard Tri-Carb 2100 TR beta counter. Data are reported as mean values of n = 3 independent experiments performed in triplicates.

PBS, RPMI and BSA fatty acid free was ordered from Sigma-Aldrich, Switzerland. Ultima Gold liquid scintillation cocktail was purchased from Perkin Elmer, Switzerland. AquaSil™ silanization fluid was purchased from Thermo Fisher Scientific, USA.

Determination of the endocannabinoid levels

Cells were seeded to Petri dishes in 5 million/5 ml culture medium/Petri dish density, and were treated with VMD11 or vehicle for 24 hours (5 ml medium/dish). At the end of the treatment, equal amount (i.e. 5 ml) of ice-cold methanol was added to the cultures, and the samples (cells and their supernatants together) were harvested and immediately stored at -80°C for further analysis.

The samples, which contained both cells and their supernatants were homogenized in a solution containing 5 pmol of $[^2H]_8$-AEA and 50 pmol of $[^2H]_5$-2-AG, $[^2H]_4$-PEA and $[^2H]_2$-OEA. The lipid-containing organic phase was pre-purified by open-bed chromatography on silica gel (Bisogno et al., 1997) and analyzed by LC-APCI-MS (Marsicano et al., 2002). AEA, 2-AG, PEA and OEA amounts (pmol) were normalized per mg of extracted lipids.

Assessment of the FAAH-activity

Sebocytes were trypsinized, and following determination of the cell count, and removal of the supernatant, they were transferred to -80°C for further analyses.

Cell pellet homogenates in 50 mM Tris-HCl, pH 7.4 were incubated for 30 min at 37°C with [^{14}C]AEA (2 μM) and with test compounds, VDM11, URB 597, UCM 707. [^{14}C]Ethanolamine produced from [^{14}C]AEA hydrolysis was measured by scintillation

counting of the aqueous phase after extraction of the incubation mixture with 2 vol. of $CHCl_3/CH_3OH$ 2:1 (by vol.).

Statistical analysis

Data were analyzed by Origin Pro Plus 6.0 software (Microcal, Northampton, MA, USA), using Student's two tailed, two samples *t*-test and $P<0.05$ values were regarded as significant differences. Graphs were plotted by using Origin Pro Plus 6.0 software (Microcal).

SUPPLEMENTARY DISCUSSION 1 – <u>Alternative interpretation of differential effects of EMT- and FAAH-inhibitors</u>

Since, at least in some cells, the possibility that EMT-inhibitors also block the release of eCBs cannot be excluded (Chicca et al., 2012), one should also take into consideration that the effects of VDM11 were generated via intracellular signaling pathways.

Indeed, there is a possibility that, in case of human sebocytes, under homeostatic conditions, rather than being up-taken, eCBs are transported out of the cells by EMT. EMT-inhibitors would block this process, and as a consequence, eCBs would be degraded more intracellularly leading to a FAAH-mediated increase in the cytoplasmic arachidonic acid (AA; an important precursor of several pro-inflammatory lipid mediators; Bennett and Gilroy 2016) level. AA has been previously shown to greatly increase lipogenesis (see e.g. Géczy et al. 2012). Following this line, one may speculate that lipogenic action of VDM11 could be mediated by the elevation of the intracellular AA levels, whereas lack of URB597's efficiency could be explained by the lack of such AA-elevation.

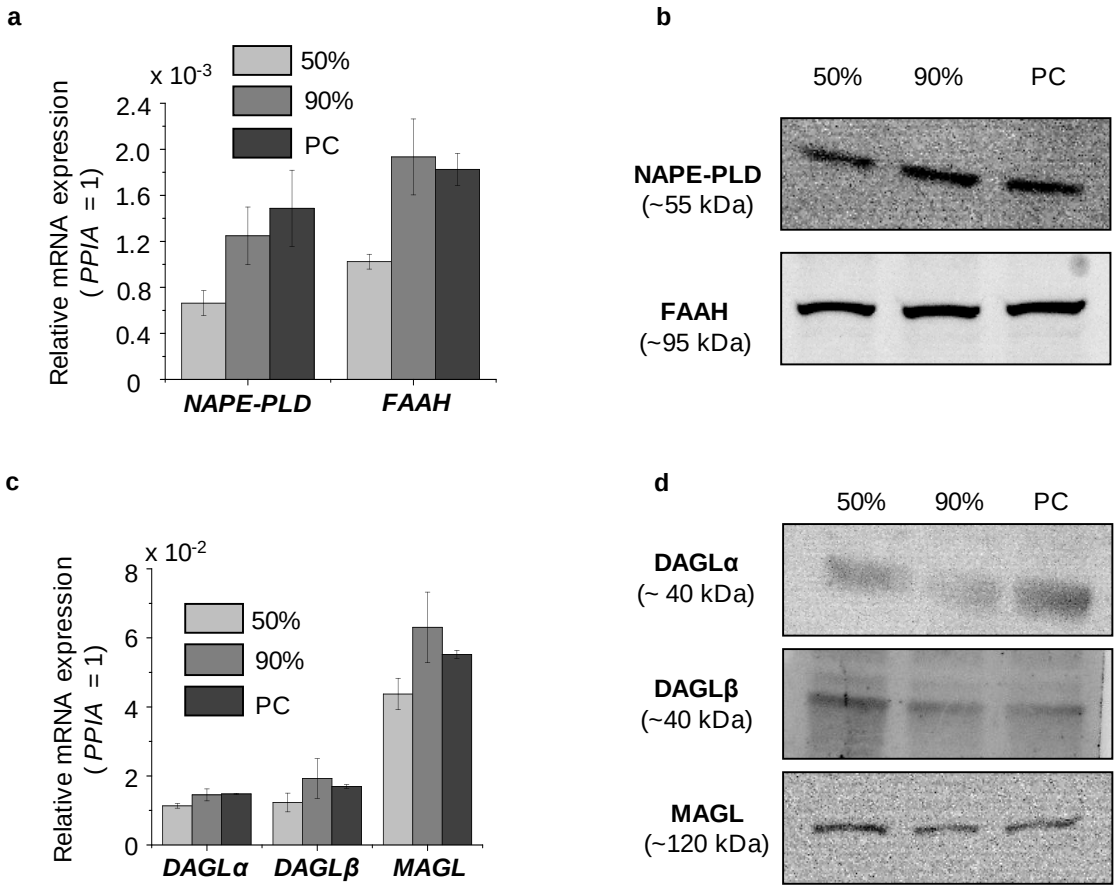
Although this hypothesis is very intriguing, several lines of evidence go against it. First, we have previously found that AEA's lipogenic effect was CB_2-dependent (Dobrosi et al. 2008), and AA does not activate CB_2 (Di Marzo 2008). Second, cyclooxygenase and lipoxygenase metabolites of AA are more likely to exert pro-inflammatory effect (Bennett and Gilroy 2016), but we showed that VDM11 suppressed LPS-induced pro-inflammatory response (**Figure 3**). Third, if exogenously applied AEA indeed needed to

be intracellularly converted to AA in order to promote SLP, VDM11 should have prevented its lipogenic action, but it had no effect on it (**Supplementary Figure S6**).

SUPPLEMENTARY DISCUSSION 2 – Possible targets of PEA and OEA in the SGs

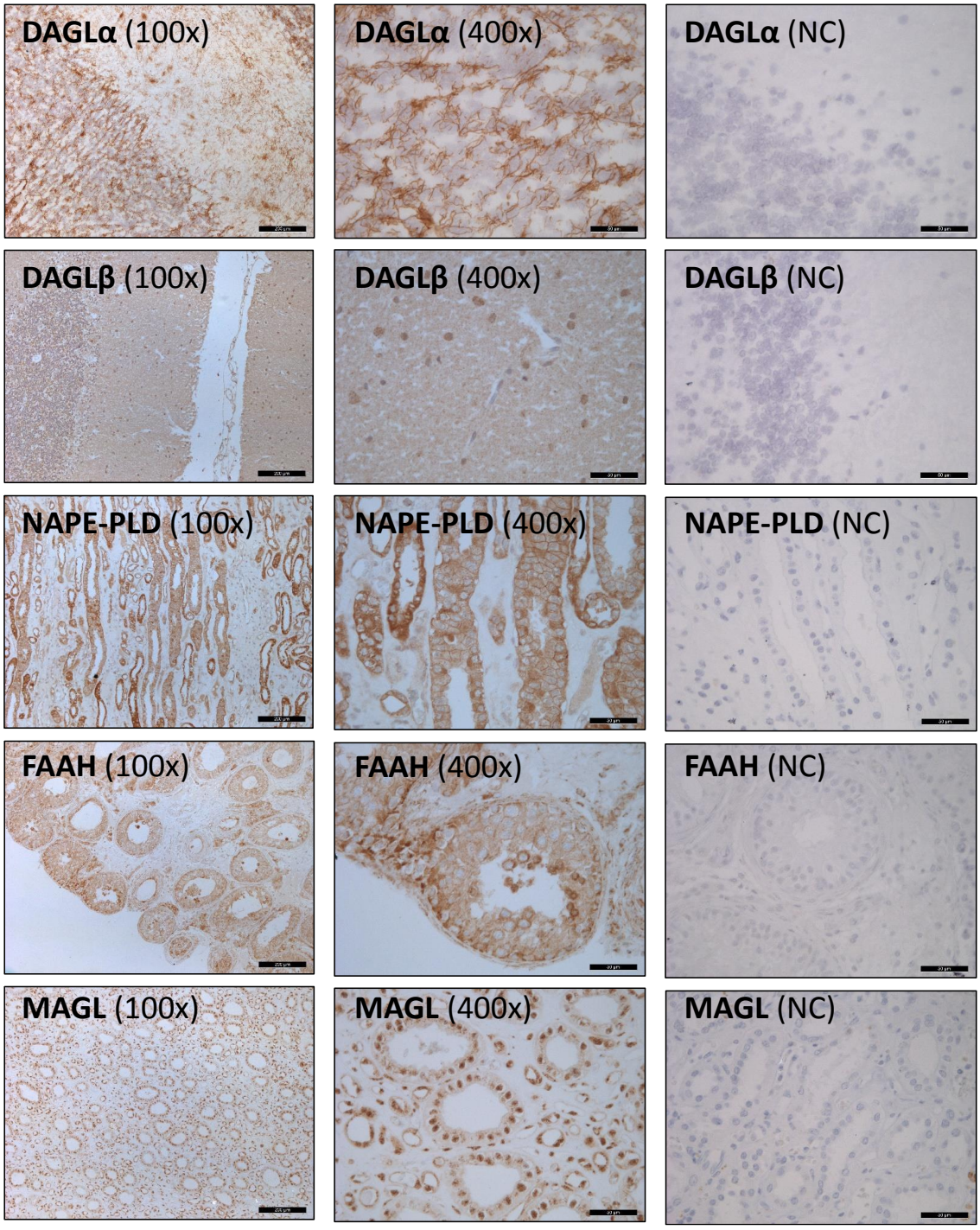
Our results also point towards new, exciting possibilities with respect to the "*cutan*nabinoid" system. Since our data argue for that not only AEA and 2-AG, but also PEA and OEA are metabolized in human sebocytes, one may speculate that these molecules might regulate yet un-identified, novel signaling pathways through activation of e.g. peroxisome proliferator-activated receptors (PPAR) or the recently de-orphanized GPR55 and GPR119 receptors (Godlewski et al. 2009; Maccarrone et al. 2015) in human sebocytes. Although the role of PPARs in regulating SG biology is already well-described (Dobrosi et al. 2008; Dozsa et al. 2016; Dozsa et al. 2014; Rosenfield et al. 1998), the expression of GPR55 and GPR119 is still unknown. Systematic studies are therefore needed to investigate the expression and putative role of these novel receptors in the biology of human SGs and, in a wider sense, in cutaneous (patho)physiology, with special emphasis on the development of inflammation-accompanied diseases such as AD.

Supplementary Figures



Supplementary Figure S1 *Major enzymes of the endocannabinoid metabolism are expressed in SZ95 sebocytes*

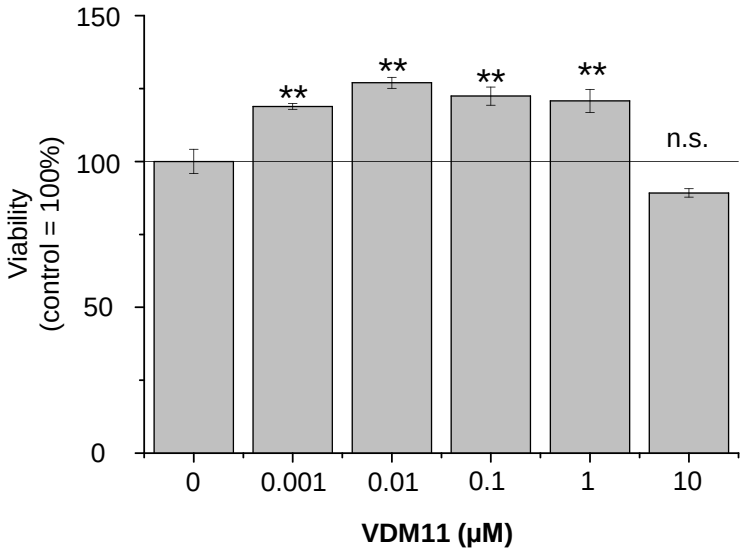
(**a** and **c**) Q-PCR on SZ95 sebocyte samples harvested at different confluences. Data of *NAPE-PLD, DAGLα* and *–β,* as well as *MAGL* and *FAAH* mRNA expressions were normalized to the level of peptidylprolyl isomerase A (*PPIA*) of the same sample, and are expressed as mean±SD of three determinations. One additional experiment yielded similar results. (**b** and **d**) Western blot analysis of lysates of SZ95 sebocytes (pc: post-confluent culture). **DAGL:** diacylglycerol lipase; **FAAH:** fatty acid amide hydrolase; **MAGL:** monoacylglycerol lipase; **NAPE-PLD:** *N*-acyl phosphatidylethanolamine-specific phospholipase D.



Supplementary Figure S2 *Optimization of the histological methods: labeling of the positive control tissues*

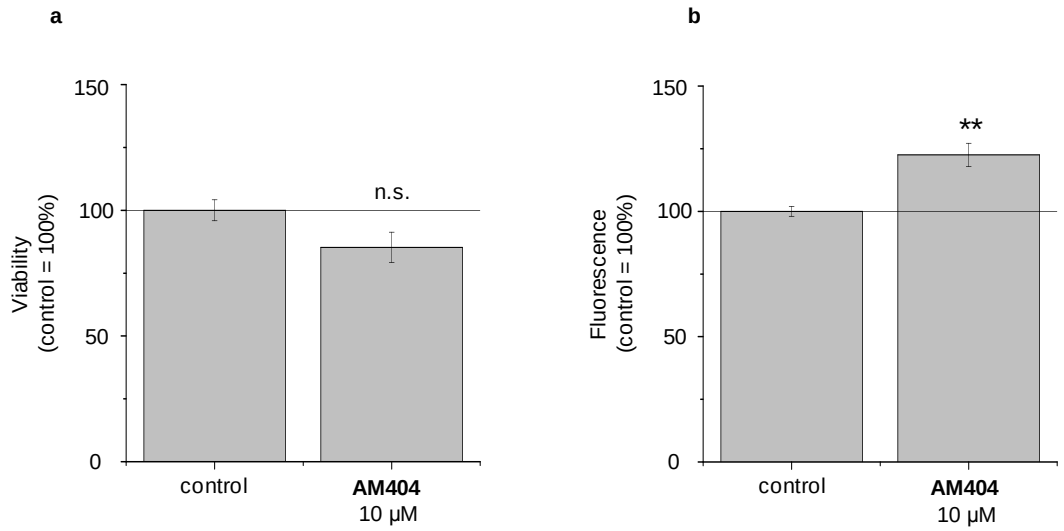
Immunohistochemistry of appropriate human tissues (**DAGL*α*** and **–β:** cerebellum; **FAAH:** testicle; **MAGL** and **NAPE-PLD:** kidney) was performed as described in the

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). 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.



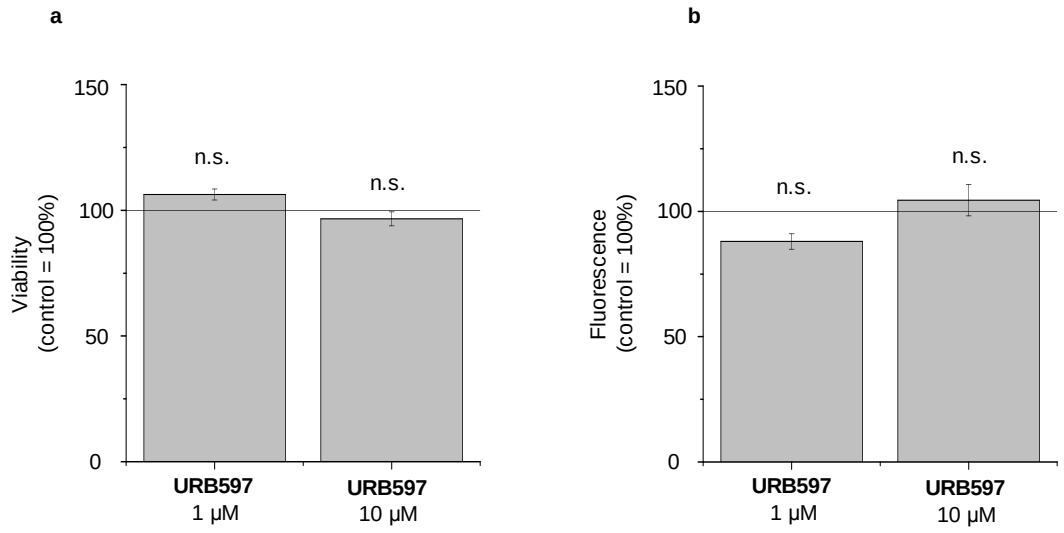
Supplementary Figure S3 *Up to 10 μM, VDM11 did not decrease viability of human sebocytes*

Viability of SZ95 sebocytes was monitored by MTT-assay 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. ** marks significant (P<0.01) difference compared to the vehicle control. n.s.: not significant difference. (When applied at higher concentrations, VDM11 was cytotoxic; data not shown.)



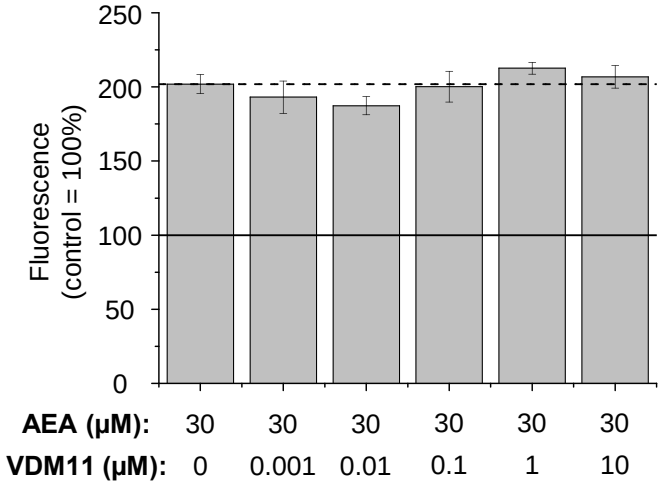
Supplementary Figure S4 *Similar to VDM11, a non-cytotoxic concentration of AM404 moderately, but significantly increases sebaceous lipid synthesis*

(**a**) Viability of SZ95 sebocytes was monitored by MTT-assay 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; n.s.: not significant difference. (**b**) 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. ** marks significant (P<0.01) difference compared to the vehicle control.



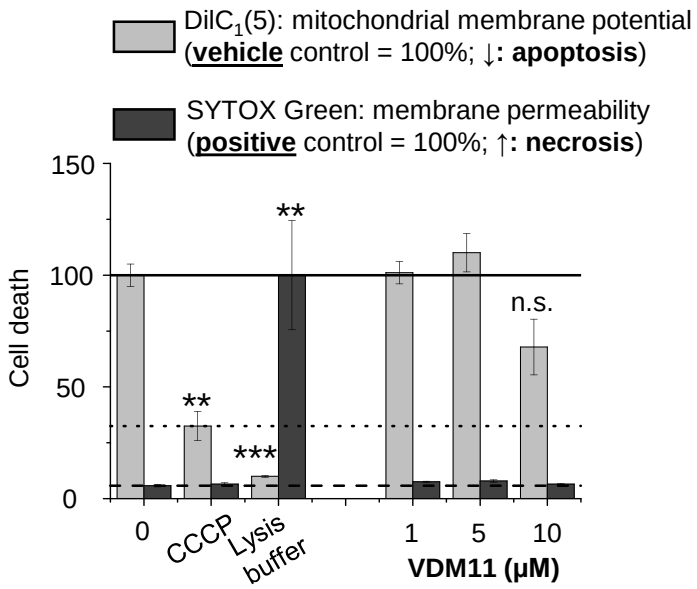
Supplementary Figure S5 *In contrast to the effects of the EMT-inhibitors, URB597 does not influence sebaceous lipid synthesis*

(**a**) Viability of SZ95 sebocytes was monitored by MTT-assay 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; n.s.: not significant difference. (**b**) 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. ** marks significant (P<0.01) difference compared to the vehicle control.



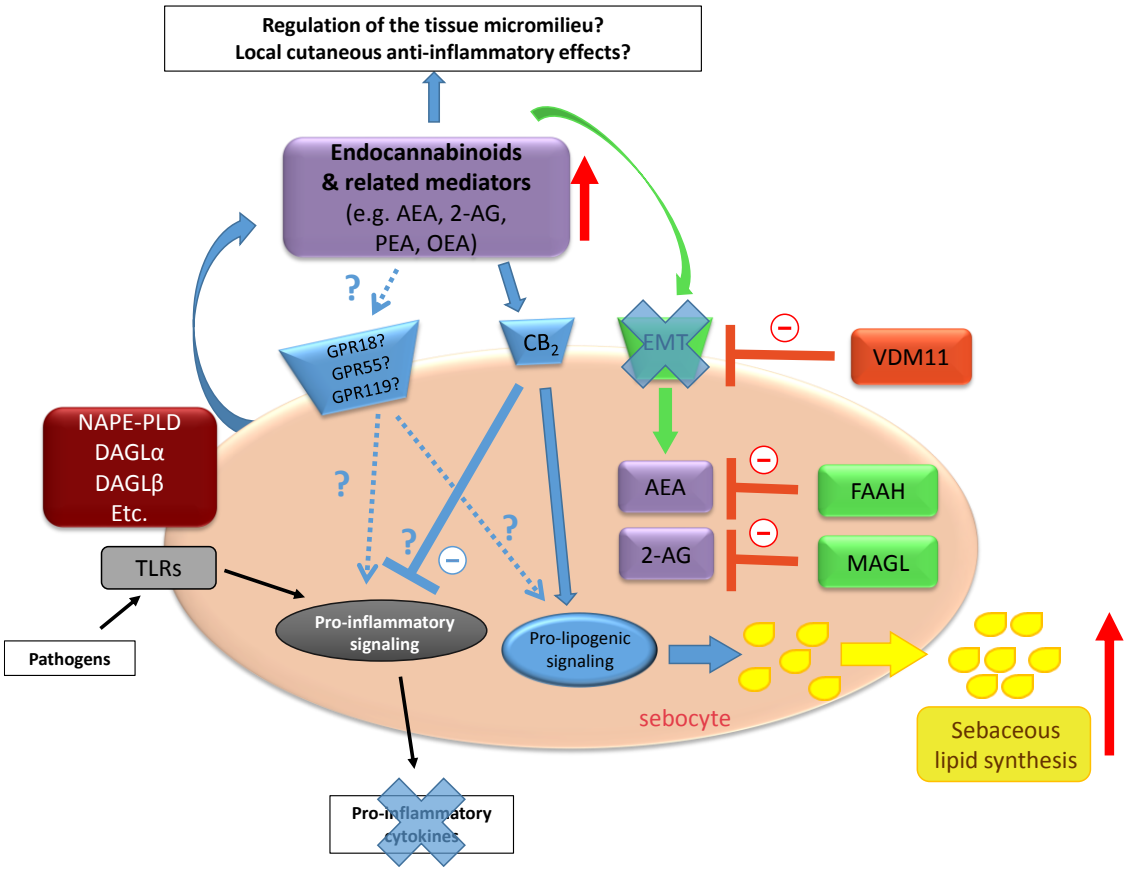
Supplementary Figure S6 *The AEA uptake inhibitor VDM11 does not further increase lipogenic action of AEA*

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. Dashed line: signal intensity in the case of AEA-treatment.



Supplementary Figure S7 *The most effective lipogenic concentration of VDM11 tends to decrease mitochondrial membrane potential*

Apoptotic and necrotic cell death of SZ95 sebocytes was investigated by $DilC_1(5)$-SYTOX Green double labeling following the indicated 48-hr treatments. 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). In general, selective decrease of $DilC_1(5)$ intensity indicates the development of "pure" apoptotic processes, whereas simultaneous increase of SYTOX Green level points to necrosis. Results are expressed in the percentage of the vehicle or positive controls ($DilC_1(5)$ and SYTOX Green, respectively) 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. n.s.: not significant difference.



Supplementary Figure S8 *Overview of the human sebaceous glands' endocannabinoid system, and the effects of VDM11*

Sebocytes express the major synthesizing (NAPE-PLD, DAGLα and –β) and degrading (MAGL, FAAH) enzymes of the eCB metabolism, and they metabolize several "classical" eCBs (AEA and 2-AG) as well as related acylethanolamides (PEA and OEA). Elevation of their levels by administration of VDM11 leads to a moderate increase of the sebaceous lipid production, and remarkable anti-inflammatory actions. Although AEA and 2-AG were shown to act through a CB_2-coupled signaling pathway leading to PPAR activation (Dobrosi et al, 2008), expression of the cellular targets of PEA and OEA (e.g. GPR55 and GPR119) are still unknown in human sebocytes.

2-AG: 2-arachidonlglycerol; **AEA:** *N*-arachidonoylethanolamine (anandamide); **CB_2:** cannabinoid receptor 2; **DAGL:** diacylglycerol lipase; **eCB:** endocannabinoid; **EMT:** (putative) endocannabinoid membrane transporter; **FAAH:** fatty acid amide hydrolase; **GPR55** and **GPR119:** G protein-coupled receptor 55 and 119 (recently de-orphanised

receptors of PEA and OEA, respectively); **MAGL:** monoacylglycerol lipase; **NAPE-PLD:** *N*-acyl phosphatidylethanolamine-specific phospholipase D; **OEA:** oleoylethanolamide; **PEA:** palmitoylethanolamide; **PPAR:** peroxisome proliferator-activated receptor; **TLR:** Toll-like receptor; **VDM11:** EMT-inhibitor.

Supplementary Tables

Supplementary Table S1: *Comparative assessment of FAAH-inhibitory capabilities of selected EMT- and FAAH-inhibitors on human SZ95 sebocytes*

Compounds	**Inhibition %** **(n=6)**
URB597 0.1 μM	66.1 ± 5.2
VDM11 5 μM	22.0 ± 1.9
VDM11 10 μM	20.9 ± 4.5
VDM11 25 μM	-8.5 ± 0.8 (stimulation)
UCM707 5 μM	4.9 ± 0.4
UCM707 10 μM	26.4 ± 4.1
UCM707 25 μM	20.1 ± 3.5

URB597: reference FAAH-inhibitor; **UCM707** and **VDM11:** EMT-inhibitors

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