Inhibitors of Dipeptidyl Peptidase IV and Aminopeptidase N Target Major Pathogenetic Steps in Acne Initiation

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Acne is a chronic disease hallmarked by sebaceous hyperplasia, follicular hyperkeratosis, and inflammation. Parallel targeting of these factors is required to treat acne effectively. Inhibitors of dipeptidyl peptidase IV (DP IV) and aminopeptidase N (APN) show strong anti-inflammatory effects on immune cells and therapeutic efficacy in autoimmune disorders. Our investigation focused on the expression and functional relevance of these ectopeptidases in three cell types which exhibit an altered phenotype in early acne lesions. We showed for the first time expression of DP IV and APN on human sebocytes. In the SZ95 sebocyte cell line, the DP IV inhibitors Lys[Z(NO_2)]-thiazolidide and Lys[Z(NO_2)]-pyrrolidide and the APN inhibitors actinonin and bestatin suppressed proliferation, enhanced terminal differentiation, and slightly decreased total neutral lipid production. The anti-inflammatory and differentiation-restoring cytokine IL-1 receptor antagonist was significantly upregulated in SZ95 sebocytes and the HaCaT keratinocyte cell line in the presence of inhibitors. Furthermore, the inhibitors suppressed proliferation and IL-2 production of *Propionibacterium acnes*-stimulated T cells *ex vivo* and enhanced the expression of the immunosuppressive cytokine transforming growth factor-β1. Our data provide first evidence for a functional role of DP IV and APN in the sebaceous gland apparatus and for their inhibitors, used alone or in combination, as completely new substances possibly affecting acne pathogenesis in a therapeutic manner.

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INTRODUCTION

Acne vulgaris is the most common skin disease worldwide. Intense medical treatment is needed in acne patients to prevent physical or psychological scarring, and annual costs for health care in acne exceed $1 billion in the US alone (Stern, 2000). Treatment efficacy is significantly enhanced by the combination of various agents to target the major pathogenetic factors in parallel (Gollnick *et al.*, 2003; Leyden, 2003); and the use of isotretinoin affecting four major pathogenetic factors is clinically limited owing to teratogenicity and side effects (McLane, 2001). Thus, the development of new therapeutic strategies overcoming the current limitations is clinically required.

Ectopeptidases are ubiquitously expressed and have pleiotropic functions (Antczak *et al.*, 2001). Interaction with agonistic antibodies or inhibitors revealed that ectoenzymes, beyond their proteolytic activity, influence fundamental biological processes such as growth, apoptosis, differentiation, adhesion, motility, invasion, cell–cell interaction, angiogenesis, and transformation (De Meester *et al.*, 1999; Iwata and Morimoto, 1999; Riemann *et al.*, 1999).

This implies a possible role of these enzymes as targets to influence pathophysiologic conditions. The pharmacological inhibition of dipeptidyl peptidase IV (DP IV) (EC 3.4.14.5., DP IV, CD26) as well as aminopeptidase N (APN) (EC 3.4.11.2, membrane alanyl-aminopeptidase (mAAP), APN, CD13) affects growth, cytokine production, and typical functions of human peripheral T cells both *in vitro* and *in vivo* (Reinhold *et al.*, 1997, 2002; Lendeckel *et al.*, 1999).

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Abbreviations: A, actinonin; APN, aminopeptidase N; B, bestatin; DP IV, dipeptidyl peptidase IV; IL-1RA, interleukin-1 receptor antagonist; LZNP, Lys[Z(NO_2)]-pyrrolidide; LZNT, Lys[Z(NO_2)]-thiazolidide; PA/P. acnes Propionibacterium acnes; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; TGF-β_1, transforming growth factor β_1; 13-cis-RA 13-cis-retinoic acid

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Inhibitors of DP IV have potent immunosuppressive and anti-inflammatory effects in various disease models such as murine experimental autoimmune encephalomyelitis (Steinbrecher *et al.*, 2001), collagen- and alkyldiamone-induced arthritis (Tanaka *et al.*, 1997), and rat cardiac transplantation (Korom *et al.*, 1997), and are presently undergoing clinical phase II and III trials (McIntosh *et al.*, 2005) for type II diabetes.

APN inhibitors have shown therapeutic efficacy in analgesia models and tumor neoangiogenesis (Fournié-Zaluski and Roques, 2002), and bestatin (B) is clinically used as an immunomodulator in cancer patients (Fujisaki *et al.*, 2003).

The skin represents an interesting new target organ of ectopeptidase inhibitors, not only because skin diseases involve the activation of immune cells but also because both DP IV and APN are expressed on human keratinocytes *in vivo* and upregulated in hyperproliferative skin diseases, for example, psoriasis (Hunyadi *et al.*, 1993; Novelli *et al.*, 1996). Previous investigations showed that inhibitors of DP IV and APN suppress keratinocyte proliferation *in vitro* (Reinhold *et al.*, 1998; Gabrilovac *et al.*, 2004) and partially restored keratinocyte differentiation *in vivo* (Thielitz *et al.*, 2004).

Thus multifactorial diseases hallmarked by hyperproliferation and inflammation such as acne are promising research fields for therapeutic applications of ectopeptidase inhibitors.

Our knowledge about the pathogenetic steps leading to acne initiation is still incomplete and subject of controversial discussion (Zouboulis *et al.*, 2005). The beginning of microcomedone formation is associated with aberrant differentiation of the follicular epithelium (Kurokawa *et al.*, 1988; Norris and Cunliffe, 1988), vascular endothelial cell activation, and inflammatory events (Jeremy *et al.*, 2003), which supports the hypothesis that acne may represent a genuine inflammatory disease. The sequence of events initiating ductal hypercornification is not yet elucidated, and hypotheses implicate changes in sebum lipid composition (Downing *et al.*, 1986), androgenic stimulation (Thiboutot *et al.*, 1997), and immunologic stimulation in keratinocytes triggered by *Propionibacterium acnes* (*P. acnes*) (Nagy *et al.*, 2005).

Future acne treatment should focus on targeting the very early steps of acne lesion development and thus normalize the primary pathogenetic factors to induce microcomedones, which are hyperproliferation of sebocytes and consecutive hyperseborrhea and hyperproliferation of follicular keratinocytes (Gollnick, 2003) as well as inflammatory processes such as specific immune responses toward *P. acnes* antigens (Mouser *et al.*, 2003) or intrinsic generation of inflammation within the sebaceous glands (Zouboulis *et al.*, 2003, 2005).

The aim of our present work was to investigate the expression and functional relevance of DP IV and APN as a new target complex on three cell types implicated in acne pathogenesis to lay the foundation of inhibitor-based future acne therapy.

RESULTS

Expression of DP IV, CD26 and APN, CD13 on human sebocytes

Using the human-immortalized sebaceous gland cell line SZ95 to assess the effects of inhibitors on human sebaceous function, we show for the first time at the levels of mRNA (Figure 1a), protein (Figure 1b and c), and enzymatic activity (DP IV = Gly-Pro pNA hydrolyzing activity 37 ± 9 pkat/10^6 cells and APN = Ala-pNA hydrolysing activity 205 ± 54 pkat/10^6 cells) that the ectopeptidases DP IV/CD26 and APN/CD13 are expressed on human sebocytes. The DP IV activity of SZ95 sebocytes was found to be 6-fold higher than that of resting peripheral blood T cells (6 ± 2 pkat/10^6 cells; Kähne *et al.*, 1999) and 2-fold higher than that of T cells stimulated for 48 hours with phytohemagglutinin (PHA), whereas APN activity exceeded that of PHA-activated T cells (31.4 ± 2.8 pkat/10^6 cells; Lendeckel *et al.*, 1999) by 6- to 7-fold. Flow cytometric studies (Figure 1b) and direct immunofluorescence staining (Figure 1c) revealed high surface expression of both ectopeptidases, with 98 ± 1% CD26-positive cells and 100% CD13-positive cells.

The immunohistochemical staining of human scalp sections demonstrated a positive, but slightly different expression pattern of both ectopeptidases in sebaceous glands; CD26 is generally stronger expressed and mainly localized in basal low-differentiated sebocytes in the periphery of the glands

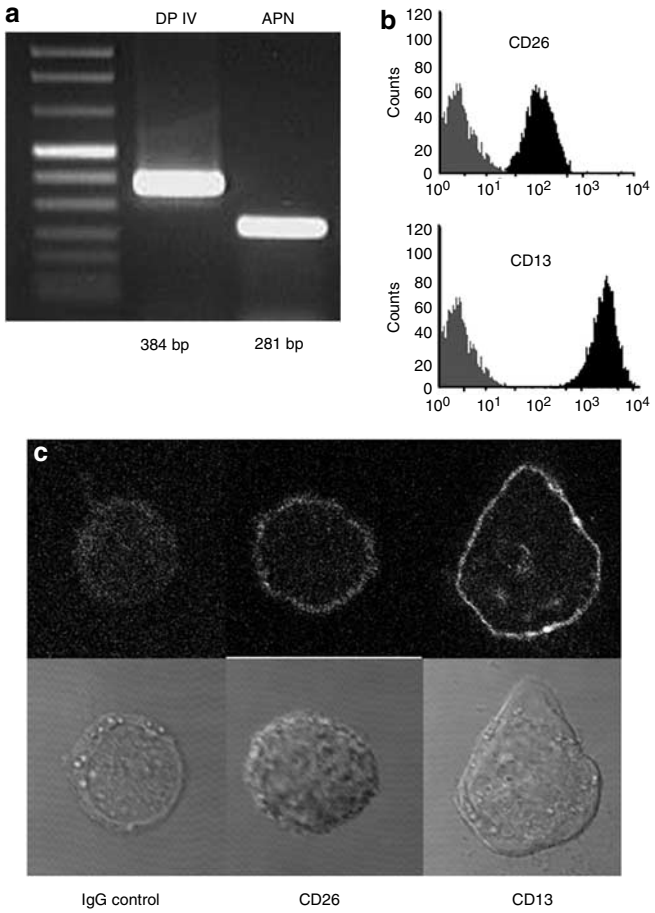

Figure 1. Expression of DP IV/CD26 and APN/CD13 on human SZ95 sebocytes. (**a**) mRNA expression of DP IV and APN. Enzymatic amplification was performed on cDNA derived from 70 to 80% confluent cell cultures. (**b**) CD26 and CD13 surface expression detected by flow cytometry. (**c**) Direct immunofluorescence staining (top) and light microscopic control (bottom) of vital SZ95 sebocytes attached to poly-L-lysine-coated cover slides.

(Figure 2i and l), whereas CD13 presents a more diffuse staining pattern in the periphery (Figure 2h and k) as well as in clusters in the center of the glands, which have been described previously (Cunliffe, 1992) as highly proliferating cells and represent undifferentiated cells sitting on the base of septa separating the lobules of the gland. Furthermore, positive expression could be shown in the epidermis (Figure 2b and c), the hair follicle (Figure 2e and f) as well as in smooth musculature (Figure 2h and i).

Inhibitors of DP IV and APN suppress enzyme activity and DNA synthesis of SZ95 sebocytes *in vitro*

The DP IV inhibitors Lys[Z(NO_2)]-thiazolidide (LZNT) and Lys[Z(NO_2)]-pyrrolidide (LZNP), and the APN inhibitors actinonin and B, were capable of significantly ($P<0.001$) suppressing enzymatic activity in a dose-dependent manner (Figure 3a and b). No significant differences could be detected between inhibitors of either DP IV or APN.

The DNA synthesis of viable SZ95 sebocytes after 48 hours incubation in the presence of different inhibitor concentrations was rapidly and dose-dependently ($P<0.0001$) suppressed (Figure 3c and d) compared to control cultures without inhibitor, which correlated with the reduction in enzyme activity. The retinoid with the most potent effects on the proliferation of sebocytes, 13-*cis*-retinoic acid (13-*cis*-RA), significantly suppressed SZ95 proliferation at 10^{-5} and 10^{-6} M ($P<0.001$), but not at 10^{-7} M (Figure 3c).

The antiproliferative effect was partly enhanced in an additive manner by the combination of inhibitors of DP IV and APN, which is demonstrated for the combination of LZNT and the most potent single inhibitor, actinonin (Figure 3d). Table 1 summarizes the inhibitory concentration of 25% suppression of DNA synthesis calculated from the mean of 6 different experiments (or inhibitory concentration of 75% suppression of DNA synthesis calculated from the mean of 6 different experiments, respectively) and inhibitory concentration of 50% suppression of DNA synthesis calculated from the mean of 6 different experiments values of SZ95 obtained for the single and combined inhibitors, compared to HaCaT, primary keratinocytes, and *P. acnes*-stimulated peripheral blood mononuclear cells (PBMCs).

Possible cytotoxic side effects of the inhibitors in the concentrations used could be excluded using the lactate dehydrogenase cytotoxicity detection assay, and by trypan blue staining, which demonstrated more than 95% viable cells in all culture systems 6 hours after inhibitor incubation (data not shown).

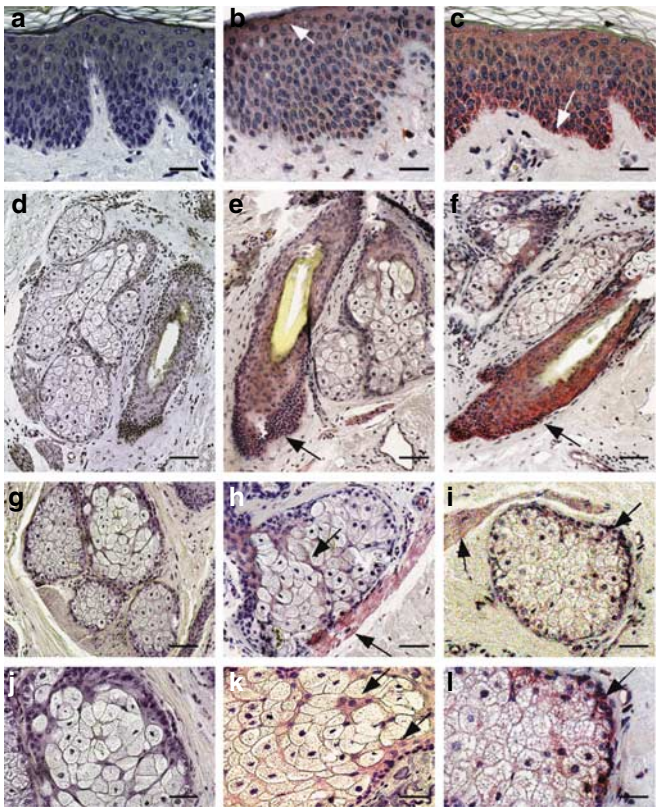

Figure 2. Detection of CD26- and CD13-positive cells in the pilosebaceous follicle. Deparaffinized sections of human scalp preteated with Proteinase K were stained with (**a**–**j**, left panel) an IgG-control or (**b**–**k**, middle panel) the anti-CD13 antibody My7 or (**c**–**l**, right panel) the anti-CD26 antibody BA5 and visualized by the immunoperoxidase technique (red, immunoreactivity). Arrows indicate (**b**, **c**) positive labelling in the epidermis, (**e**, **f**) the hair follicle, (**h**, **i**) smooth musculature, and (**h**, **i**, **k**, **l**) within the sebaceous glands. (**a**–**c**, **j**–**l**) Bar = 0.1 mm and (**d**–**f**, **g**–**i**) 0.2 mm.

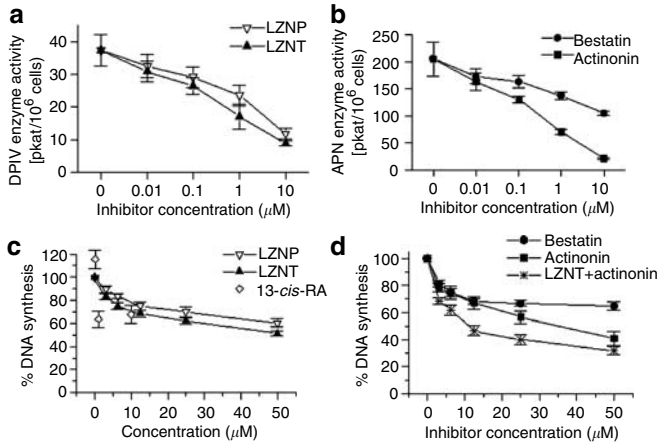

Figure 3. Suppression of enzymatic activity and proliferation of SZ95 sebocytes in the presence of inhibitors. (**a**) and (**c**) The inhibitors of DP IV LZNP and LZNT dose-dependently ($P<0.001$) (**a**) reduce enzymatic activity (Gly-Pro-pNA hydrolysis) and (**c**) DNA synthesis ($P<0.001$) measured by [^{3}H]thymidine incorporation. (**b**) and (**d**) The APN inhibitors actinonin and B suppress both the (**b**) enzymatic activity (Ala-pNA hydrolysis) and (**d**) DNA synthesis in a dose-dependent manner ($P<0.001$). The enzymatic activity represents the mean ± SEM of four independent experiments; statistics were calculated using the General linear model procedure of SAS 8.2. The DNA synthesis values are expressed as percentage of [^{3}H]thymidine incorporation in relation to control cultures without inhibitor (100% = 16,047 ± 1,773 c.p.m.) and represent the mean ± SEM of six independent experiments. (**c**) 13-*cis*-RA significantly suppressed DNA synthesis compared to control cultures (0 μM) at 10 and 1 μM ($P<0.001$), but not at 0.1 μM. The retinoid effect was not dose-dependent.

Table 1. DP IV and APN inhibitors suppress DNA synthesis in different cell types

	SZ 95		HaCaT		PKC		PBMC (PA)	
Inhibitor	IC_{25}[1]	IC_{50}[2]	IC_{25}[1]	IC_{50}[2]	IC_{25}[1]	IC_{50}[2]	IC_{50}[2]	IC_{75}[3]
LZNP	5	92	12	>100	22	—	6	15
LZNT	2.5	55	8	55	20	100	3	7.5
Actinonin	2.5	36	6.5	23	4	—	4	—
Bestatin	3	—	37	—	21	—	3	6
LZNP+A	1.25	12	2.5	12	3	>100	1.25	2.5
LZNT+A	1	11	2.5	10	2.5	50	1.25	2
LZNP+B	1.25	20	8	>100	3	>100	1.25	3.5
LZNT+B	1.25	15	6.25	32	3	58	1.75	2

A, actinonin; APN, aminopeptidase N; B, bestanin; DP IV, dipeptidyl peptidase IV; LZNP, Lys[Z(NO_2)]-pyrrolidide; LZNT, Lys[Z(NO_2)]-thiazolidide; PA, *P. acnes*; PKC, primary keratinocyte; PBMC, peripheral blood mononuclear cell.
[1]IC_{25}=inhibitory concentration of 25% suppression of DNA synthesis calculated from the mean of six different experiments.
[2]IC_{50}=inhibitory concentration of 50% suppression of DNA synthesis calculated from the mean of six different experiments.
[3]IC_{75}=inhibitory concentration of 75% suppression of DNA synthesis calculated from the mean of six different experiments.

Inhibitors of DP IV and APN induce terminal differentiation in SZ95 sebocytes

Sebocyte differentiation is characterized by increasing cell size, granularity, and lipid content. To assess inhibitor effects on sebaceous differentiation, we used a flow cytometric Nile Red fluorescence assay, which indicated the neutral lipid content per cell. We showed that incubation of SZ95 sebocytes with inhibitors for 48 hours produces a dose-dependent increase in sebocyte differentiation (Figure 4a). The effects on terminal differentiation correspond well with the antiproliferative effects of the single inhibitors, with actinonin as single inhibitor and a combination of LZNT and actinonin showing the greatest capacity to influence both parameters. 13-*cis*-RA significantly suppressed sebocyte differentiation only at 10^{-7} M ($P<0.05$), a concentration where the proliferation was not affected.

We then attempted to ascertain the extent to which the inhibitors influenced the neutral-lipid composition of sebocytes. Therefore, we measured the cholesterol, free fatty acids, triglycerides, wax esters, and squalene fractions of confluent SZ95 sebocytes using high-performance thin layer chromatography after an incubation period of 48 hours in the presence of each of the four inhibitors separately and a combination of the most potent inhibitors, at a concentration of 50 μM. Squalene was not detectable in our system, and the amounts of triglycerides and wax esters were low, which indicates that the differentiation grade of cultured sebocytes is lower as compared to freshly isolated cells. Despite the increase of single-cell differentiation seen in the Nile Red assay, the total neutral lipid content of the cultured cells showed a trend for a decrease (Figure 4b), which was not statistically significant. The relative composition of neutral lipids (Figure 4b) as well as free fatty acids analyzed by gas chromatography (data not shown) was not significantly altered in inhibitor-treated cultures; however, a trend for a decrease in the (saturated) free fatty acid fraction was

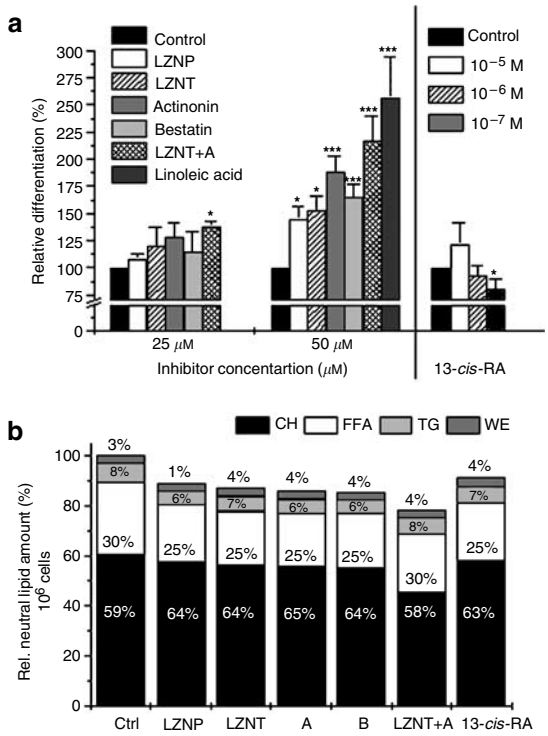

Figure 4. Influence of DP IV and APN inhibitors on sebocyte differentiation and neutral lipid composition. (**a**) Inhibitor-induced (left part) or 13-*cis*-RA-induced (right part) dose-dependent relative increase of terminal differentiation per cell for SZ95 sebocytes measured by Nile Red fluorescence intensity detected after 48 hours of incubation. The data are demonstrated relatively to control (without inhibitor or retinoid) and represent the mean ± SEM of seven independent experiments. Linoleic acid served as positive control (*$P<0.05$, ***$P<0.001$, Dunnett's multiple comparison test). (**b**) Reduction of total neutral lipid content and relative composition of neutral lipids extracted from confluent SZ95 cell cultures in the presence of DP IV and APN inhibitors at 50 μM or of 13-*cis*-RA at 10^{-7} M after 48 hours of incubation. Values are demonstrated relatively to control and represent the mean of six experiments. WE, wax esters; TG, triglycerides; FFA, free fatty acids; CH, cholesterol.

detectable in the presence of inhibitors. In the presence of 13-*cis*-RA tested as a control substance at concentrations 10^{-5}–10^{-7} M, no change in the neutral lipid amounts after 48 hours could be detected. Figure 4b includes the concentration with the strongest effect on lipid production (10^{-7} M, concentrations 10^{-5} and 10^{-6} M not shown), which is corresponds to the results obtained in the differentiation assay (Figure 4a).

DP IV and APN inhibitors enhance IL-1RA production in sebocytes and keratinocytes

Sebocytes are able to produce proinflammatory cytokines such as tumor necrosis factor-alpha, IL-1α, and IL-1β (Boehm *et al.*, 1995). Therefore, we investigated inhibitor effects on the production of these proinflammatory cytokines and the antagonist interleukin-1 receptor antagonist (IL-1RA) in SZ95 sebocytes, and we additionally measured IL-1RA production of HaCaT and primary keratinocytes.

In SZ95 sebocyte supernatants, very low amounts of IL-1α (maximum 17 pg/ml), and IL-1β (maximum 4 pg/ml) were found, whereas tumor necrosis factor-alpha was not detectable. The amounts of these cytokines were not significantly changed in the presence of the inhibitors (data not shown). However, we found a strong expression of IL-1RA protein in control supernatants (1,118 $\pm$ 204 pg/ml), which was slightly but significantly increased in the presence of each of the four inhibitors (1.3- to 1.7-fold at 50 μM; 1.7- to 2.4-fold at 100 μM, data not shown) after 48 hours incubation ($P<0.05$). Similar results were obtained at a lower base level (153 $\pm$ 35 pg/ml) in HaCaT keratinocytes (1.3- to 1.7-fold increase at 50 μM, 1.5- to 2.4-fold at 100 μM, data not shown).

To further investigate the regulation of IL-1RA, we applied the most potent single inhibitors LZNT and actinonin (50 μM each) as well as their combination and measured the IL-1RA protein levels and mRNA levels at different time points after the inhibitor was added. We found a time-dependent increase of IL-1RA on the protein level ($P<0.001$). Comparing the inhibitor effects at the different time points, we found a trend for an additive effect of the inhibitor combination, comparable to the observed effects on proliferation demonstrated in Table 1 and Figure 3d; however, a statistically significant difference between the combination and the most potent single inhibitor could be demonstrated only in HaCaT keratinocytes on the mRNA level 3 hours after inhibitor incubation (Figure 5d).

In general, the effect was maximum after 48 hours on the protein levels in SZ95 sebocytes (Figure 5a) and HaCaT keratinocytes (Figure 5b), and maximum after 3 hours on the mRNA levels, with increases of up to 4-fold in SZ95 sebocytes (Figure 5c) and 2.6-fold in HaCaT keratinocytes (Figure 5d).

Inhibitors of DP IV and APN suppress DNA synthesis and IL-2 production of *P. acnes*-stimulated PBMC and induce TGF-β1 production *in vitro*

PBMC from 16 donors with or without a history of acne showed a significantly increased but varying proliferative response after stimulation with *P. acnes* antigen (Figure 6a),

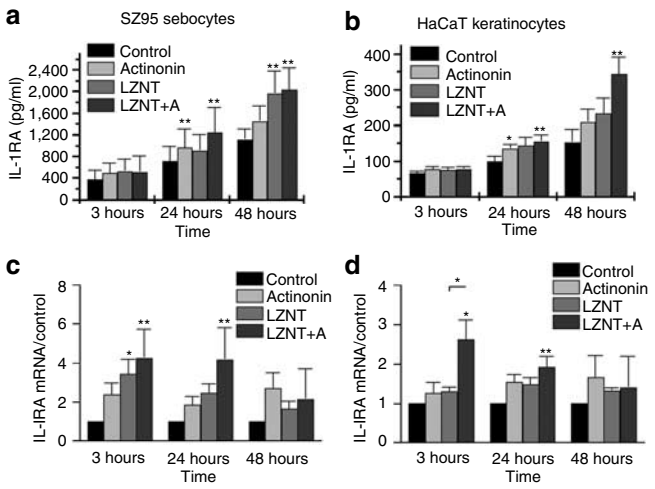

Figure 5. DP IV and APN inhibitors increase IL-1RA expression in SZ95 sebocytes and HaCaT keratinocytes. (**a, b**) Increase of IL-1RA protein expression in supernatants of (**a**) SZ95 sebocytes and (**b**) HaCaT keratinocytes at 3, 24, and 48 hours after inhibitor incubation. The data represent the mean $\pm$ SEM of four independent experiments. Statistics were calculated separately at each time point, asterisks indicate significance compared to control without inhibitor (*$P<0.05$; **$P<0.01$ Tukey's multiple comparison test). (**c, d**) Increase of IL-1RA mRNA amounts detected by quantitative real-time PCR in (**c**) SZ95 sebocytes and (**d**) HaCaT keratinocytes. The bars represent the mean $\pm$ SEM of six independent experiments. The data are presented as relative amounts compared with the control or as indicated (*$P<0.05$, **$P<0.01$ Tukey's multiple comparison test).

which was even in less responding subjects slightly increased as compared to unstimulated controls, probably indicating a previously described weak mitogenic effect of *P. acnes* antigen (Jappe *et al.*, 2002). PBMC from different donors showed equal rates of DNA synthesis after stimulation with the mitogen PHA, which were higher in comparison to those obtained with antigen-stimulated cells.

Furthermore, we studied the effects of the DP IV and APN inhibitors on *P. acnes* antigen-stimulated DNA synthesis of subjects showing a markedly increased proliferative response (stimulation index >5). In accordance to previous data obtained from mitogen-stimulated PBMC and T cells, we found strong dose-dependent ($P<0.001$) antiproliferative activities for each of the four inhibitors or combinations of a DP IV and APN inhibitor used in a concentration range of 1–20 μM on *P. acnes* antigen-stimulated PBMC (Figure 6b and d). Moreover, we demonstrated a significant decrease in IL-2 production ($P<0.01$) and an increase in the production of the immunosuppressive cytokine transforming growth factor β_1 (TGF-β1) after 24 hours in the presence of each DP IV and APN inhibitor used in concentrations of 10 μM (Figure 6c). With regard to the cytokine production, the combination showed no significant additive effect as compared to the single inhibitors.

DISCUSSION

The design of an ideal future acne treatment is subject of controversial discussion (Zouboulis *et al.*, 2005) because our

knowledge about the relative contribution of various factors to acne initiation is still incomplete. Based on clinical experience supported by the efficacy of oral isotretinoin and combination therapy, parallel targeting of sebaceous hyperplasia, follicular hyperkeratosis, and inflammation represents the most promising approach to treat acne.

The presented data demonstrate that inhibitors of the ectopeptidases DP IV or APN have the capacity to influence exactly these major pathogenetic factors of acne *in vitro*. They profoundly affect proliferation and cytokine balance of three cell types with observed alterations in early acne pathogenesis by antagonizing hyperproliferation of sebocytes leading to hyperseborrhea, hyperproliferation of keratinocytes inducing follicular hyperkeratosis, and proliferation of immune cells being part of a specific immune response toward *P. acnes* antigen.

We showed for the first time that DP IV and APN are highly expressed on the surface of human SZ95 sebocytes *in vitro*, suggesting that sebocytes could be effectively targeted by peptidase inhibitors. As animal models in acne failed to predict pharmacological outcomes in humans, we used the immortalized human sebaceous gland cell line SZ95 for the investigations on human sebaceous function. *In vivo* expression was detected by immunohistochemistry of human scalp sections and revealed positive staining, especially in undifferentiated, highly proliferating (Cunliffe, 1992) sebocytes at the periphery of the sebaceous lobules, which have been considered to be the ''regenerative pool'' of the glands. Previous research revealed that the skin expression of both enzymes is not constant but regulated in an age-dependent manner (Sorrell *et al.*, 2003). Thus, the next experimental step will be to further investigate the differential expression of the ectopeptidases in inflammatory acne lesions to delineate pathophysiological significance and better predict the possible therapeutical efficacy *in vivo*. This is of particular interest because the ectopeptidase neutral endopeptidase (NEP; CD10; EC 3.4.24.11), which is often coexpressed with DP IV and APN *in vivo* (Riemann *et al.*, 1999), has been reported to be significantly upregulated in acne patients but was not detectable in sebaceous glands of healthy subjects (Toyoda *et al.*, 2002).

Our results demonstrate that inhibitors of both DP IV/CD26 and APN/CD13 significantly suppress enzyme activity and SZ95 sebocyte proliferation in a dose-dependent manner. Comparable effects could be obtained at the same time point with 13-*cis* RA only in concentrations of 10^{-6} M or higher (Figure 3c).

The suppression of proliferation is inversely correlated with an induction of terminal differentiation of sebocytes, which is characterized by increasing size, accumulation of lipid droplets, and nuclear degeneration (Wróbel *et al.*, 2003). It is conceivable that the balance between antiproli-

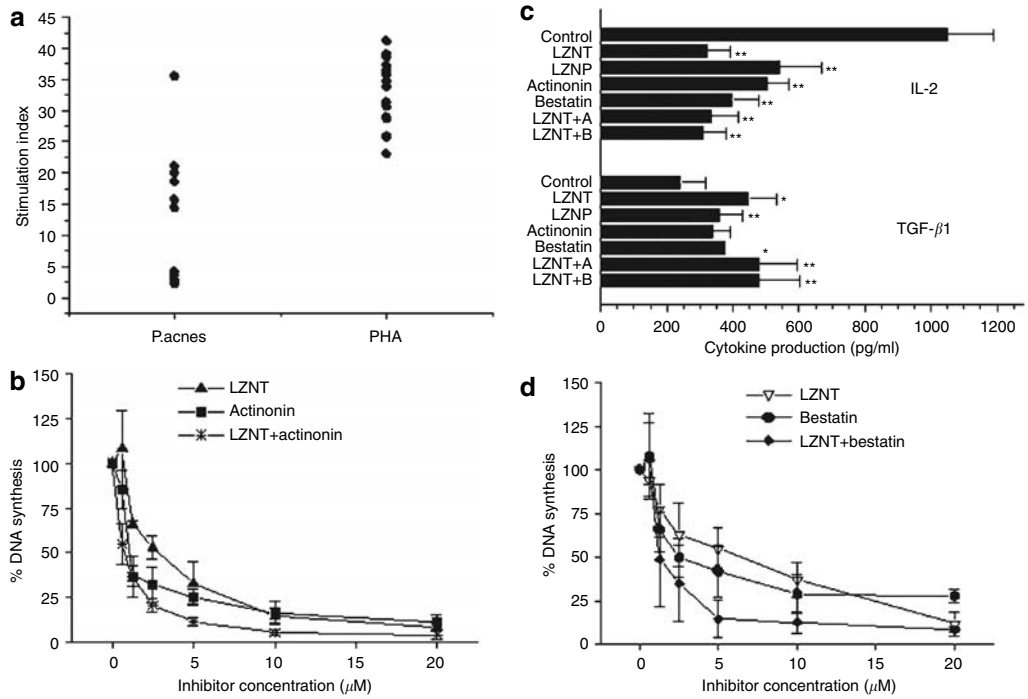

Figure 6. Effects of inhibitors on proliferation and cytokine production of *P. acnes* antigen-stimulated PBMCs. (**a**) Stimulation indices of *P. acnes* antigen-stimulated PBMCs compared to mitogen (PHA)-stimulated PBMCs of 16 adult subjects. (**b, d**) Dose-dependent suppression ($P<0.001$) of DNA synthesis of *P. acnes* antigen-stimulated PBMCs in the presence of DP IV and APN inhibitors and their combination at concentrations of 0.62–20 μM. [^{3}H]thymidine incorporation is indicated as mean ± SD of six independent experiments. Values are expressed as percentage of [^{3}H]thymidine incorporation in relation to control cultures without inhibitors (100% = 8,986 ± 3,109 c.p.m.). (**c**) Absolute inhibitor-induced changes in cytokine levels detected in PBMC supernatants after 24 hours at a concentration of 10 μM: increase of latent TGF-β1 and suppression of IL-2 relative to control cultures without inhibitors. Values represent the mean ± SD of three different experiments. Statistics were calculated related to the control (*$P<0.05$, **$P<0.01$, Dunnett's multiple comparison test).

ferative and prodifferentiative effects will not induce hyperseborrhea, because despite an increase in the single-cell lipid content by enhanced differentiation (Figure 4a), the total neutral lipid collected from cells cultivated for 2 days in the presence of inhibitors tends to decrease after a short incubation period (Figure 4b). It is tempting to speculate that the block in cellular proliferation of undifferentiated cells leads to an accelerated normal differentiation process, which ends up with apoptosis/holocrine secretion. This is also corroborated by increased nuclear fragmentation indices, which we observed in the presence of inhibitors (data not shown). Interestingly, 13-*cis*-RA induced no significant decrease in differentiation at concentrations with a marked antiproliferative effect, which further supports the hypothesis that both processes might be inversely related. The significance of these inhibitors effects on the complex human sebaceous function *in vivo* remains to be established.

The proinflammatory cytokine IL-1α represents another hallmark in early acne pathogenesis. Increased levels can be found in uninvolved follicles, early acne lesions (Aldana *et al.*, 1998; Jeremy *et al.*, 2003), and abundantly in extracted mature comedones (Ingham *et al.*, 1992). Its functional relevance was studied in an *in vitro* organ model, where it induced a follicular hyperkeratosis in isolated sebaceous infundibula (Guy *et al.*, 1996). This process could be blocked or reversed by the application of IL-1RA, indicating that the ratio of IL-1α/IL-1RA is involved in regulating growth and differentiation of keratinocytes. It is likely that keratinocytes are one major source of the increased IL-1 levels around acne follicles, which might be induced by *P. acnes* or *P. acnes* heat-shock proteins (Graham *et al.*, 2004). Sebocytes ''stressed'' *in vitro* produced IL-1α on the mRNA and protein level (Zouboulis *et al.*, 1998), and *in vivo* investigations detected presence of IL-1α immunoreactivity and mRNA in sebaceous glands (Anttila *et al.*, 1992; Boehm *et al.*, 1995). We demonstrated that inhibitors of DP IV and APN slightly but significantly increase the expression of the anti-inflammatory cytokine IL-1RA in sebocytes and keratinocytes, both being possible sources of IL-1α *in vivo*. Variations in the IL1-RA/IL-1α ratio during keratinocyte cell cycle (Hammerberg *et al.*, 1998) would provide one possible mechanism for our previously observed inhibitor effects on keratinocyte proliferation and differentiation *in vitro* and *in vivo* (Thielitz *et al.*, 2004).

Interestingly, simultaneous inhibition of both enzymes exhibited partially additive effects on the proliferation, but not cytokine production, in different cell types (Figures 3d and 6b, d; Table 1). With regard to the development of therapeutic agents, the application of an inhibitor combination might be useful, because synergistic effects have been reported on T cells *in vitro* (Lendeckel *et al.*, 2003) and *in vivo* (Bank *et al.*, 2006; Biton *et al.*, 2006).

The molecular basis of antiproliferative and immunomodulating effects of DP IV or APN inhibitors are only partly understood (von Bonin *et al.*, 1998; Lendeckel *et al.*, 1999, 2000; Reinhold *et al.*, 2005). One important observed mechanism in T cells, the release of TGF-β1 4–6 hours after inhibitor treatment (Kähne *et al.*, 1999), leads to an arrest in the cell cycle at G1 (Polyak *et al.*, 1994) and partly explains the observed suppression of T-cell proliferation, which we could also demonstrate in *P. acnes*-stimulated PBMC.

The pathophysiologic role of T cells found in uninvolved follicles before any detectable signs of keratinocyte hyperproliferation is yet unclear, but their further accumulation in acne lesion progress (Layton *et al.*, 1998) in coincidence with other histologic hallmarks of immunologic/vascular activation strongly suggests a functional contribution to disease aggravation. Within the increased perifollicular CD4+ T-cell population, the majority consisted of memory/effector (CD45RO) cells, with a similar proportion exhibiting a skin-homing phenotype (Jeremy *et al.*, 2003), suggesting a specific antigenic response. Human T-cell clones isolated from lesional acne skin showed a significant increase in proliferation and IFN-γ production in response to *P. acnes* stimulation, which could not be achieved with psoriatic T-cell lines or in presence of other skin commensals (Mouser *et al.*, 2003). Our results demonstrate that inhibitors of DP IV and APN strongly suppress the T-cell proliferation in response to *P. acnes* antigenic stimulation of PBMC and additionally shift the cytokine production toward an anti-inflammatory phenotype.

In summary, our results provide first *in vitro* evidence for inhibitors of DP IV and APN as a new substance class with the capacity to influence three major pathogenetic factors of acne: sebaceous hyperplasia, follicular hyperkeratosis, and (early) inflammation.

The presented data suggest that the single or parallel inhibition of DP IV and APN might represent a promising and novel therapeutic strategy for this global disease. Clinical trials using newly designed highly specific inhibitors will be necessary to confirm these intriguing findings *in vivo*.

MATERIALS AND METHODS

All described studies using materials obtained from human subjects were conducted according to the Declaration of Helsinki Principles and approved from the medical ethical committee of the Otto-von-Guericke University, Magdeburg, Germany. Participants gave their written informed consent.

Cell culture conditions

PBMCs were isolated by density-gradient centrifugation over Ficoll Paque gradients as described previously (Reinhold *et al.*, 1997) and seeded at a density of 10^5 cells/100 μl in serum-free AIM-V medium (Invitrogen, Karlsruhe, Germany). They were stimulated with heat-inactivated (80°C, 30 minutes) *P. acnes* protein suspensions in a concentration of 10 μg/ml, obtained from the *P. acnes* strain DSM 20458 (DMSZ Braunschweig, Germany) cultured for 3 days in differential clostridial broth (Merck, Darmstadt, Germany) to reach stationary growth phase. The culture was centrifuged at 16,000 × g at 4°C and the pellet resuspended in phosphate-buffered saline (Biochrom Berlin, Germany) for protein determination. Unstimulated PBMC and PHA-stimulated (PHA, 1 μg/ml, Abbott, Wiesbaden, Germany) PBMC served as controls.

HaCaT keratinocytes were maintained in serum-free medium (Keratinocyte-SFM, Invitrogen, Karlsruhe, Germany) and SZ95 sebocytes (Zouboulis *et al.*, 1999) in Sebomed Basal Medium

(Biochrom) supplemented with 10% heat-inactivated fetal bovine serum, antibiotics, 5 ng/ml human epidermal growth factor (Promocell, Heidelberg, Germany), and $CaCl_2$ (Sigma-Aldrich, Deisenhofen, Germany) to obtain a final concentration of 1 mM. All experiments using peptidase inhibitors were performed in serum-free medium (Sebomed Complete Medium, Biochrom). Actinonin and B were obtained from Sigma-Aldrich (Deisenhofen, Germany). LZNP and LZNT were synthesized by K. Neubert and J. Faust. Cell viability was determined using the lactate dehydrogenase cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany) at time points of 4 and 6 hours after inhibitor incubation and by trypan blue (Sigma-Aldrich) staining.

RNA preparation from SZ 95 and HaCaT and qualitative RNA amplification

Total RNA from 1×10^6 cells was prepared using the RNeasy Kit provided by Qiagen (Hilden, Germany) following the recommended protocol. The resulting RNA was quantified spectrophotometrically using a GeneQuant (Pharmacia, Freiburg, Germany), aliquoted, and stored ethanol-precipitated at −80°C until further use.

One microgram of total RNA was transcribed in a 20 μl reaction mixture containing 20 U of avian myeloblastosis virus reverse transcriptase (Promega, Mannheim, Germany) in the supplied buffer, 0.5 mmol/l desoxynucleoside triphosphates, 10 mmol/l random hexanucleotides (Boehringer, Mannheim, Germany), and 50 U of placenta RNase inhibitor (Ambion, Austin, USA).

Reverse transcription reaction (1.2 μl) was used as template for amplification. Forty cycles were performed in a Peltier thermal cycler PTC-200 (Biozym Diagnostik, Hessisch Oldendorf, Germany) in 50 μl reaction buffer containing 2.5 U HotStarTaq™ DNA Polymerase (Qiagen, Hilden, Germany), 200 μM dNTP, and 0.5 μM of the corresponding RT primer set of APN (upstream, 5′-gTCTACTg CAACCTATCg; downstream, 5′-gATggACACATgTgggCACC) and DP IV (upstream, 5′-gATgCTACAgCTgACAgTCgC; downstream, 5′-Tgg TgACCATgTgACCCACTg) obtained from BioTeZ (Berlin, Germany). The initial activation step at 95°C for 15 minutes was followed by 40 cycles of denaturing for 1 minutes at 94°C, annealing for 0.5 minutes at 60°C, and extension at 70°C for 1 minute. The reaction was stopped after a final extension step at 72°C for 5 minutes and products visualized on a 1.5% agarose gel.

RNA quantification

Quantitative PCR was performed using the iCycler (real-time PCR device, Bio-Rad, Munich, Germany). For IL-1RA determination, the 25 μl reaction mixture consisted of 12.5 μl QuantiTect™-SYBR-Green™-Master-Mix (Qiagen, Hilden, Germany), 1 μl of the reverse transcription reaction, and 0.5 μmol/l of the specific primers for IL-1RA (upstream, 5′-gggAgAAAATCCAgCAAg; downstream, 5′-TgA CACAggACAggCACAT) synthesized by Jenabioscience (Jena, Germany). Initial denaturation and activation of *Taq* polymerase at 95°C for 15 minutes was followed by 42 cycles with denaturation at 94°C for 30 seconds, annealing at 55°C for 48 s, and elongation at 72°C for 36 s. Amounts of 18S mRNA were determined using the RT primer pair available from Ambion and used to normalize cDNA contents. The fluorescence intensity of the double-strand-specific SYBR-Green I, reflecting the amount of actually formed PCR product, was read real-time at the end of each elongation step. Data are given as arbitrary units normalized to 18S RNA amounts. All samples were analyzed in triplicate.

Immunofluorescence staining of cells

Immunofluorescence staining of SZ 95 cells was performed using the monoclonal anti-CD13 antibody WM15 and the monoclonal anti-CD26 antibody clone M-A261 (both mIgG1, BectonDickinson Biosciences, Heidelberg, Germany). For control of nonspecific labelling, we tested the binding of an irrelevant IgG1 monoclonal mouse antibody (BectonDickinson Biosciences). Labelled cells were analyzed by flow cytometry (FACS-Calibur, BectonDickinson Biosciences) and visualized after attachment to poly-L-lysine-coated cover slides using the confocal laser scan microscope Leica TCS SP2 (Leica Camera AG, Solms, Germany).

Immunohistochemistry

Deparaffinized sections from 6-mm human scalp punch biopsies were incubated with Proteinase K solution (DakoCytomation GmbH, Hamburg, Germany) for 1 minutes, washed three times in Tris-buffered saline solution containing 0.05% Tween 20, pH 7.6, and consecutively stained with the monoclonal antibodies My7 (anti-CD13, mIgG1, 1:25, BeckmannCoulter, Krefeld, Germany, 60 minutes) and BA5 (anti-CD26, mIgG2a, 1:25, BeckmannCoulter, 30 minutes). Negative controls included incubation with an isotype-specific control (mIgG, 1:25, BeckmannCoulter, 30 minutes) and omission of the first antibody.

The consecutive steps were performed using standard immunoperoxidase techniques following the protocol of the LSAB-2 System-AP staining kit (DakoCytomation). Slides were incubated for 20 minutes in the dark with the chromogenic substrate Fast RED, rinsed with and then placed for 10 minutes in distilled water, and finally counterstained with Mayer's hematoxylin (10 seconds) before mounting in Faramount Aequous Mounting Medium (DakoCytomation).

Enzymatic assay

Enzymatic activity of APN was determined in triplicate in phosphate-buffered saline, pH 7.4, using Ala-4 nitroanilide (Ala-pNA, Sigma-Aldrich, Deisenhofen, Germany) as chromogenic substrate. Ala-pNA (100 μl) were added in a concentration of 5 mM to cellular suspensions of 10^5 cells/100 μl. Reactions were stopped by adding 900 μl acetate buffer (1 M, pH 4.4) either immediately (control) or after 60 minutes of incubation at 37°. After centrifugation (2 minutes; 10,000 × g), the absorbance of the supernatant was detected spectrophometrically at 392 nm. Enzymatic activity of DP IV was determined after 120 minutes of incubation using 3 mM Gly-Pro-4-nitroanilide (Gly-Pro-pNA, Sigma-Aldrich) added to cellular suspensions.

Proliferation assay

HaCaT keratinocytes, SZ95 sebocytes (1×10^5 cells/ml), and PBMCs (1×10^6/ml) were seeded in 96-well plates (Nunc, Wiesbaden, Germany) in serum-free medium. PBMCs obtained from 16 donors were stimulated either with *P. acnes* protein (10 μg/ml) or PHA (1 μg/ml) or left unstimulated. The stimulation index was calculated as mean counts per minute (c.p.m.)/c.p.m. of unstimulated control cultures. Different concentrations of the inhibitors (3.125–50 μM) actinonin, B, LZNT, and LZNP or a combination of DP IV and APN

inhibitors were added after 24 hours to adherent cells or directly to PBMCs (1.25–20 μM). 13-*cis*-RA (Sigma-Aldrich, Deisenhofen, Germany) was added to SZ95 sebocytes under dimmed yellow light at concentrations of 10^{-5} 10^{-7} M. After further 48 hours (keratinocytes and sebocytes), 4 days (PBMCs stimulated with PHA) or 7 days (PBMCs stimulated with *P. acnes* protein), the cultures were pulsed for additional 6 hours with [^{3}H]methyl-thymidine (0.2 μCi/well; ICN, Meckenheim, Germany). The incorporated radioactivity harvested on glass fiber filters was measured by scintillation counting.

Cytokine assays

For the generation of supernatants, 10^5 cells/ml (keratinocytes, sebocytes) and 10^6 cells/ml (PBMCs) were seeded in 24-well plates (Nunc, Wiesbaden, Germany). Inhibitors were added directly to PBMC in concentrations of 10 μM. Adherent cells (keratinocytes and sebocytes) were allowed to attach for 24 hours before the inhibitors were added in concentrations of 50 or 100 μM. Supernatants were collected 4, 24, and 48 hours after the inhibitor was added. IL-1β, tumor necrosis factor-alpha, TGF-β1, IL-2, and IL-1RA were measured using commercially available ELISA kits (R&D Systems, Wiesbaden, Germany). IL-1α was determined using an EIA kit obtained from PromoCell (Heidelberg, Germany).

Nile Red sebocyte differentiation assay

A subconfluent layer of 5×10^5 SZ95 sebocytes was incubated for 48 hours in the presence of inhibitors in concentrations of 25 and 50 μM or 13-*cis*-RA at concentrations of 10^{-5}–10^{-7} M. Cells were dissociated with trypsin, incubated for 10 minutes with 1 μg/μl Nile Red (Sigma-Aldrich, Deisenhofen, Germany), washed twice with phosphate-buffered saline, and analyzed immediately by standard flow cytometric techniques using the FACSCalibur (BectonDickinson, Heidelberg, Germany). Linoleic acid (Sigma-Aldrich) was dissolved in ethanol to give a final concentration of 100 μM in medium with 0.1% ethanol.

Lipid extraction and high-performance thin-layer chromatography assessment of lipid fractions

SZ95 sebocytes were seeded into 6-cm Petri dishes (5×10^5 cells/dish). Subconfluent cultures were incubated with inhibitors at a concentration of 50 μM in serum-free medium for 48 hours or 13-*cis*-RA at 10^{-5}–10^{-7} M and then washed twice with phosphate-buffered saline. Lipids were extracted with *n*-hexane/ethanol (95/5 (v/v)) under ultrasonication for 5 minutes. The extracts were filtered and evaporated twice using a vacuum centrifuge (Univapo 150H, Uniequip, Martinsried, Germany). For separation of the lipid mixture into different classes, we used aminopropyl-bonded phase columns (Supelco-Sigma, Munich, Germany). Lipid extracts were applied in a volume of 200 μl chloroform to the *n*-hexane pretreated columns. Squalene and wax esters were extracted in 3 ml *n*-hexane. Triglycerides were subsequently extracted with 2 ml *n*-hexane/methanol/chloroform (88/10/2 = v/v), free fatty acids with 3 ml diethyl ether/acetic acid (98/2), and cholesterol with 3 ml *n*-hexane/ethylacetate (95/5). All lipid fractions were dried and re-diluted in chloroform/methanol (2/1) before high-performance thin-layer chromatography analysis carried out on 20×10 ml silica-coated glass plates (Merck, Darmstadt, Germany). Samples were applied to the *n*-hexane pretreated plates using a Linomat IV (Camag, Berlin, Germany). Plates were developed to 5.5 cm in *n*-hexane/diethyl ether/acetic acid (80/20/10), dried, and subsequently developed in petroleum benzene to full plate length. The dried plates were dipped in a solution containing 10% $CuSO_4$ and 8% H_3PO_4 and charred at 180°C for 15 minutes. Densitometric quantification was performed using the CD 60 (Desaga, Heidelberg, Germany) densitometer.

Statistical analysis

In the data sets demonstrated in Figures 3 and 6b, c the effects of interest are (inhibitor) groups, dose and their interaction. In Figure 5, the effects of interest are group, time, and their interaction. These were determined by repeated measures analysis of variance using the General linear model procedure of SAS, version 8.2 (SAS Institute Inc., Minneapolis, MN).

In data sets comparing multiple (inhibitor) groups to a control group (Figures 4 and 6c), one-way analysis of variance was performed with subsequent post-tests if overall *P*-value was <0.05.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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