Regulator for oily skin and balance of skin´s microflora

Sylvia Eisenberg¹, Nicole Beyer¹, Jutta ZurLage¹, Anett Moschner¹, Hansjürgen Driller¹

1. Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany

Objective and Introduction
In modern life, image matters and consumers around the world have become aware of their appearance. Oily skin is a major issue to some because it affects those areas that are most vulnerable and exposed, like the chin, forehead and nose. Additionally, oily and impure skin causes a real aesthetic problem as it may lead to a higher susceptibility of acne development.

Alterations in the pilosebaceous unit, an association of sebaceous glands and hair follicles, are involved in acne development. Causes are increased sebum excretion, induced by e.g. stress and hormonal changes, colonization of the hair follicle by Propionibacterium acnes, alterations of lipid composition and its oxidization, and the release of inflammatory mediators into the skin.¹,²

The skin is a complex ecosystem on its own, about 1.8m² in size, providing diverse habitats for a wide range of microorganisms. A balanced microbiota is usually related to healthier skin. Disruptions in microbial populations, therefore, can be linked to cutaneous pathological states such as acne and atopic dermatitis. Modulating unbalanced populations and their interactions, between microbiome and immune system, may prevent the risk of skin disorders³, enabling a healthy and refined skin complexion.

A new efficient cosmetic active has been designed to counteract oily skin and its consequences by reducing the sebum level and maintaining the skin´s beneficial microflora. Consumers could, therefore, benefit from shine-free, clear skin and would be less susceptible to acne development and skin irritation.

Methods and Results
The cosmetic effects of the new ingredient 4-Hydroxy-cyclohexanecarboxylic acid butyl ester - CCBE - regulate oily and impure skin appearance. These effects were evaluated and proven in various in vitro and in vivo assays and studies.

Excessive sebum production can be caused by diverse mechanisms in the pilosebaceous unit, such as 5-α reductase activity in the testosterone metabolism. Herein, oily skin regulating properties were evaluated within the testosterone metabolism. To determine the reducing effect on 5-α reductase activity, a non-differentiated sebocyte cell line was used.

The reduction effect was determined on thin layer chromatography by densitometry analysis of testosterone and corresponding testosterone metabolites, such as dihydrotestosterone (DHT), 4-androstene-3,17-dione and androstanedione. Quantifications were done using the
DHT/ testosterone ratio. Studies were performed comparing the effects of CCBE to Zinc PCA, a known cosmetic substance reducing the 5-α reductase activity. Both substances were tested at the highest possible non-cytotoxic concentration. The control of the assay corresponds to 100% and the reference substance Finasteride at 10 µM treat level, a highly active pharmaceutical substance, validated the assay.

Zinc PCA, tested at 0.015mM (0.0005%), had no significant reducing effect on the 5-α reductase activity, while CCBE, tested at 0.3mM (0.006%), showed a weak, but significant decrease of DHT/ testosterone metabolism in comparison to Finasteride. CCBE reduced the 5-α reductase activity in two different *in vitro* studies by 25% and is superior to that of Zinc PCA (Figure 1).

Additionally, CCBE showed a reducing effect on production of androstanedione, which corresponds to a reduction of 5-α reductase enzymatic activity, whereas Zinc PCA had only a slight reducing effect (Table 1).

![Figure 1: Effect of compounds CCBE and Zinc PCA on DHT production in sebocyte cell line - Determination of the DHT / testosterone ratio](image)

<table>
<thead>
<tr>
<th>Test compounds</th>
<th>Concentration</th>
<th>Control (%)</th>
<th>sem (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>Finasteride</td>
<td>10 µM</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>CCBE</td>
<td>300 µM</td>
<td>73</td>
<td>3</td>
</tr>
<tr>
<td>Zinc PCA</td>
<td>15 µM</td>
<td>90</td>
<td>2</td>
</tr>
</tbody>
</table>

*Table 1: Effect of compound CCBE on androstanedione production in sebocyte cell line; sem - standard deviation*
To evaluate impacts on bacteria colonization and skin’s microflora balancing properties, the growth reduction of *Propionibacterium acnes*, *Staphylococcus epidermidis* and *Staphylococcus aureus* was evaluated within *in vitro* bacteria suspension assays and quantified by analysis of the remaining germ colonies. Bacteria suspensions comprising *Propionibacterium acnes* ATCC 6919 were incubated anaerobically at 35°C and bacteria suspensions comprising *Staphylococcus epidermidis* ATCC 12228 or *Staphylococcus aureus* ATCC 6538 were incubated aerobically at 35°C. The colony count of the bacteria suspensions was determined initially and after defined incubation times, 20 min, 60 min or 24 hours, and then expressed in CFU (Colony Forming Units) per mL.

Under the experimental conditions of the assay, CCBE, tested at 0.5% and 1.0%, showed a slight time-dependent reduction effect of *Staphylococcus epidermidis* ATCC 12228. The bacteria count was reduced from $10^5$ to $10^3$ CFU/mL. Meanwhile, Zinc PCA showed a reduction effect at 0.5% use level and a complete killing effect at 1.0% use level after 24 hours (Figure 2).

![Figure 2: Effect of compound Zinc PCA and CCBE on growth reduction of *Staphylococcus epidermidis* ATCC 12228](image)

Both, CCBE and Zinc PCA, showed a complete killing effect on *Propionibacterium acnes* ATCC 6919 at a use level of 0.5% and 1.0%. CCBE already reduced the growth of *Propionibacterium acnes* ATCC 6919 after 60 min, while Zinc PCA reached the complete killing effect only after 24 hours (Figure 3).
Figure 3: Effect of compound Zinc PCA and CCBE on growth reduction of *Propionibacterium acnes ATCC 6919*

CCBE and Zinc PCA, both tested at 0.5% and 1.0%, showed a time-dependent reduction effect on *Staphylococcus aureus ATCC 6538*. Bacteria count was reduced from $10^5$ to $10^3$ CFU/mL (CCBE) and from $10^5$ to $10^2$ CFU/mL (Zinc PCA) (Figure 4).

Figure 4: Effect of compound Zinc PCA and CCBE on growth reduction of *Staphylococcus aureus ATCC 6538*

CCBE and Zinc PCA only marginally reduced *Staphylococcus aureus* growth. Nevertheless, CCBE reduced significantly *Propionibacterium acnes* growth, whereas only slightly affecting *Staphylococcus epidermidis*. On the other hand, Zinc PCA worked less selectively, killing both, *Propionibacterium acnes* and *Staphylococcus epidermidis*, at a use level of 1.0%.
CCBE was also tested in an *in vitro* study on reconstructed human epidermis, to prove adhesion reduction of the harmful bacteria *Staphylococcus aureus*.

In this assay, the *Staphylococcus aureus* bacteria were radiolabeled by incubation with [3H]-adenine and the bacteria suspension was adjusted to 0.5 OD at 525 nm, which corresponds to a final bacteria count of $10^9$ CFU/mL. Finally, CCBE and radiolabeled bacteria were topically applied on pre-treated and pre-incubated reconstructed human epidermis and incubated for 1 hour at 37°C. After incubation, the reconstructed human epidermis was washed and the remaining radioactivity, corresponding to the adherent bacteria, was measured by liquid scintillation counting.

CCBE, tested at 1%, showed a moderate, but significant inhibitory effect on *Staphylococcus aureus* adhesion on reconstructed human epidermis. CCBE reduced *Staphylococcus aureus* adhesion by 35%. However, tested at lower concentration, CCBE did not show any significant effect (Table 2).

<table>
<thead>
<tr>
<th>Test compounds</th>
<th>Concentration</th>
<th>Inhibition</th>
<th>sem</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>0</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>CCBE</td>
<td>0.5%</td>
<td>9</td>
<td>11</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>35</td>
<td>11</td>
<td>*</td>
</tr>
</tbody>
</table>

*Table 2:* Effect of compound CCBE on the adhesion of *Staphylococcus aureus* onto reconstructed human epidermis; sem - standard deviation, ns: p > 0.05 - not significant, *: p: 0.01 to 0.05 - significant

Finally, the oily skin reducing, skin refining and anti-blemish properties of the new ingredient were proven by an *in vivo* study. The randomized and blinded *in vivo* study on acne prone skin, comprised test subjects having an oily or combined skin type with a sebum level of at least 130µg/cm² and a skin inflammation score of at least 1 corresponding to slight irritation. The O/W emulsion, containing 1% CCBE (verum - test emulsion) or not (placebo - control emulsion), was applied twice a day on the whole face over 8 weeks.

Changes in sebum level were measured on the forehead using a Sebumeter® SM 815 (Courage + Khazaka electronic GmbH, Cologne, Germany). On the mentolabial area (area between the chin and lower lip) or cheek, the follicular fluorescence was recorded and quantitatively evaluated using the Visiopor® PP34N camera (Courage + Khazaka electronic GmbH, Cologne, Germany). Skin inflammation and redness were documented via photography of the skin surface and evaluated by expert grading. In addition, the test subjects evaluated the efficacy by means of a questionnaire.
As illustrated in Figure 5, CCBE reduced the skin surface sebum significantly. The sebum level, measured on the forehead, was significantly reduced by 14% after 4 weeks, and by 35% after 8 weeks. The placebo formulation showed only a reduction of 19% after 4 and 8 weeks.

![Bar chart showing the reduction of sebum over 4 and 8 weeks for Placebo Group and Verum Group.]

Figure 5: Effect of compound CCBE on the reduction of surface sebum; Statistical evaluation according to Wilcoxon signed-rank test: * p < 0.05 – significant, ** p < 0.01 - very significant, *** p < 0.001 - extremely significant

In addition, the total number and the total area of spots were analyzed by measuring follicular fluorescence. The total number of fluorescence spots showed no difference in comparison to initial measurements (data not shown). Nevertheless, the total area of fluorescence spots against baseline was significantly reduced by 21% after 8 weeks of treatment with the test formulation (Figure 6).
Figure 6: Effect of compound CCBE on the reduction of bacterial porphyrins; Statistical evaluation according to Wilcoxon signed-rank test: $p < 0.05$ – significant, $** p < 0.01$ - very significant, $*** p < 0.001$ - extremely significant.

The reduction of fluorescence area is also clearly visible on the following photographic documentation examples showing one test subject from each group. Photographs of the test subject of the control group showed no decrease in fluorescence area (Figure 7, a-c), while photographs of the test group showed a significant reduction (Figure 8, d-f).

Figure 7: Photographic documentation of fluorescence of bacterial porphyrins: Effect of placebo emulsion - Control group (1 test subject)

Figure 8: Photographic documentation of fluorescence of bacterial porphyrins: Effect of compound CCBE - Test group (1 test subject)
Furthermore, the formulation containing CCBE reduced significantly the skin inflammation/redness score by 10% after 4 weeks, and by 23% after 8 weeks of treatment (Figure 9).

![Graph showing the reduction in skin inflammation/redness score](image)

**Figure 9**: Effect of compound CCBE on the skin inflammation score; Statistical evaluation according to Wilcoxon signed-rank test: $p < 0.05$ – significant, $^{**} p < 0.01$ - very significant, $^{***} p < 0.001$ - extremely significant

Finally, in a self-assessment of efficacy parameters, the test subjects rated each of the illustrated parameters, on a 6 point scale (1 - very good to 6 - insufficient), see Figure 10. The verum group scored the efficacy parameters related to an improved skin consistently better compared to the placebo group.

![Comparison of self assessment of several efficacy parameters](image)

**Figure 10**: Comparison of self assessment of several efficacy parameters – placebo group versus verum group presented in spider web
Discussion and Conclusion
The new ingredient CCBE lowers 5-α reductase activity as demonstrated in in vitro tests. The effect in reducing 5-α reductase activity could be shown in two transformation pathways: testosterone to DHT and 4-androstene-3,17-dione to androstanedione. However, DHT interacts mainly with the androgen receptor, expressed in the basal layer of the sebaceous gland, due to greater potency (5-10 times) than testosterone. Therefore, the reduction of 5-α reductase may lead to a decreased sebocyte proliferation and with this reduced lipid production.  

Sebaceous sites are colonized mainly by Propionibacteria and Staphylococci, where the balance between Staphylococcus epidermidis and Staphylococcus aureus is of relevance. Staphylococcus epidermidis supports skin’s own protective shield and keeps potentially pathogenic germs, like Staphylococcus aureus, under control by producing its own antimicrobial peptides. CCBE decreases the growth of Propionibacterium acnes to a notable effect. In contrast to Zinc PCA, CCBE showed a significant effect reducing Propionibacterium acnes growth, whereas only slightly affecting Staphylococcus epidermidis. Concurrently, the adhesion of the non-beneficial germ Staphylococcus aureus was significantly reduced on reconstructed human epidermis. Due to the fact that CCBE could only slightly reduce the growth of Staphylococcus aureus, the observable adhesion reduction effect cannot be influenced by germ growth reduction. As a result, CCBE may hinder the adhesion of non-beneficial germs. Thus, CCBE may help to balance a healthy and beneficial skin microflora.

The performed anti-blemish in vivo study confirmed that CCBE is able to reduce the surface sebum level and the colonization of Propionibacterium acnes. The development of inflammatory metabolites (porphyrins), produced by Propionibacterium acnes, was positively influenced in the verum group. CCBE reduced significantly the porphyrin fluorescence area, which was confirmed by less visible inflammation signs. As a consequence, this may contribute to a refining of the pores.

Measured data, the statistical analysis and the self-assessment of test subjects, demonstrate the efficacy of the substance.

All in all, a novel ingredient has been found with a unique mode of refining consumers’ skin complexion. It is a step towards a healthier and more aesthetic look, while promoting skin health and thus supporting the skin’s defense system.
References

(1) E. Makrantonaki et. al., Dermato-Endocrinology 2011, 3(1), 41-49: An update on the role of the sebaceous gland in the pathogenesis of acne


(3) C.T. Branco, J. P. Guimaraes, Household and Personal Care Today 2015, 10(2): Modulation of skin microbiota by topical prebiotics
