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INVESTIGATIVE REPORT

In vitro Activity of Phytosphingosines against *Malassezia furfur* and *Candida albicans**

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Long-chain sphingoid bases, e.g. phytosphingosine, sphingosine and sphinganine, main constituents of the stratum corneum, can strongly inhibit the growth of microorganisms that are known to have undesirable effects on the skin. The aim of this study was to investigate the *in vitro* activity of different phytosphingosine preparations against *Malassezia furfur*, and, in comparison, against the common facultative pathogenic yeast *Candida albicans*. An agar dilution test for minimum inhibitory concentration (MIC) investigation of phytosphingosine base, phytosphingosine lactic acid salt, phytosphingosine HCl, and phytosphingosine glycolic acid salt was carried out using D.S.T. agar containing 2% olive oil and 0.2% Tween 80, to allow growth of the lipophilic yeast. *M. furfur* growth inhibition *in vitro* could be achieved only at extremely high phytosphingosine concentrations. Phytospingosine base had the lowest MIC value (mean 6,250 µg/ml, corresponding to 0.63% of phytospingosine in the agar). For the different phytosphingosine salts – lactic acid salt, HCl and glycolic acid salt – 4–8 fold higher MIC values were noted. Unexpectedly, there was a growth stimulating effect of *Malassezia* at lower phytosphingosine concentrations. In comparison, growth of *Candida albicans* strains was inhibited at phytosphingosine concentrations between 152 and 269 µg/ml. *Key words: in vitro susceptibility testing; minimum inhibitory concentration; Staphylococcus aureus.*

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Intercellular lipids in the stratum corneum are responsible for the barrier function of mammalian skin (1). The main components of the stratum corneum lipids are ceramides, cholesterol, and free fatty acids. There is a sequential change of lipid composition within the epidermis. At the interface between stratum granulosum and stratum corneum, the lipids undergo considerable metabolic changes: the phospholipids are degradaded into glycerol and free fatty acids, and glucosylsphingolipids into ceramides (2).

Long chain sphingoid bases, e.g. phytosphingosines (PS), sphingosines, and sphinganines are known to be present in the stratum corneum in free form but also as components of ceramides. These molecules are potent inhibitors of protein kinase C and as a consequence they seem to be involved in the differentiation of epidermal keratinocytes. There are also reports suggesting that sphingoid bases play an important role in regulating the micro-flora of the skin.

It was shown that PS, the most abundant free sphingoid base in the stratum corneum, could strongly inhibit the growth of microorganisms which are known to have undesirable effects on the skin. Application of PS will result in a healthy skin flora. It is known that, even at very low concentrations, PS can completely inhibit growth of most Gram-positive bacteria, but more importantly also Gram-negative microorganisms, such as *Escherichia coli* and *Pseudomonas aeruginosa*.

Superficial mycoses such as cutaneous candidosis, dermatophytosis, pityriasis versicolor, and some related infections are common diseases and are generally confined to the stratum corneum in the epidermis and cutaneous appendages. *Malassezia furfur* (formerly *Pityrosporum ovale*) and other *Malassezia* species are not only the causative agents for pityriasis versicolor, but probably also for seborrhoeic dermatitis and dandruff (3, 4).

Hitherto, the *in vitro* susceptibility of the lipid-dependent yeast *Malassezia furfur* against PS has never been examined. The aim of this study was therefore to investigate the *in vitro* activity of different PS preparations against *Malassezia furfur* strains. In comparison, the common facultative pathogenic yeast *Candida* (*C.*) *albicans* and Gram-positive bacteria of the genus *Staphylococcus* were investigated.

MATERIAL AND METHODS

Strains

Altogether, 7 different *Malassezia*-strains were isolated from different patients suffering from dandruff ($n = 3$), seborrhoeic dermatitis ($n = 2$), and pityriasis versicolor ($n = 2$). A fungal isolate was considered to be *Malassezia* if it was lipophilic and

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if the microscopical morphology conformed to descriptions of Ahearn & Simmons (5). *M. furfur* isolates were cultivated and further maintained on Sabouraud's 4%-dextrose agar (SIFIN, Berlin; pH 5.7) containing 2% olive oil and 0.2% Tween 80 at 37°C.

Precultivation

Single colonies of these isolates were subcultivated on so-called mDixon agar (6). This modified Dixon-Agar (7) has the advantage that the addition of olive oil and Tween 80 to the medium is not necessary. Here, glycerolmonostearate was used as modification.

The following composition of Dixon agar was used: malt extract agar 6% (Oxoid/Unipath, Basingstoke, Hampshire, England); ox gall 2% (Oxoid/Unipath, Basingstoke, Hampshire, England); Tween 40 1% (Merck-Schuchardt, Hohenbrunn, Germany); glycerolmonostearate 0.25% (Vaselinefabrik Wasserfuhr GmbH, Bonn, Germany); streptomycin sulphate 40 $\mu g\,ml^{-1}$ (Grünenthal GmbH, Stolberg, Germany); cycloheximide 250 $\mu g\,ml^{-1}$ (Ferak, Berlin, Germany); distilled water ad 100%.

In vitro susceptibility testing

In vitro susceptibility testing was carried out as previously described (8, 9). An agar dilution test for minimum inhibitory concentration (MIC) investigation of PS base, PS lactic acid salt, PS HCl, and PS glycolic acid salt was carried out using D.S.T. agar (Oxoid/Unipath, pH adjusted to 5.7) containing 2% olive oil and 0.2% Tween 80. All the above-mentioned agents for susceptibility testing were purchased from Cosmoferm, Delft, The Netherlands.

Heated D.S.T. agar with olive oil and Tween 80 were used to dissolve the PS. In medium without olive oil and Tween 80, solubility of the lipids was very poor and the resulting test medium showed diminished homogenicity. Serial twofold dilutions of PS ranging from 10 to 50,000 µg/ml were prepared in D.S.T. agar. To allow growth of *M. furfur*, this agar contained 2% olive oil and 0.2% Tween 80.

Yeast cell aliquots were prepared as suspensions with olive oil, sterile distilled water and Tween 80 (3:5:2). These suspensions were vortexed twice for 20 s. The yeast cell suspension (approximately 10^8 cfu/ml) was inoculated onto media using a multipoint inoculator, which delivered 3–4 µl per spot, resulting in a final concentration of $3–4 \times 10^5$ cfu. Results were recorded after incubation at 37°C for 96 h. The lowest drug concentration at which a strain showed no growth was considered to be the MIC. To rule out any inhibitory effect of D.S.T. medium to the tested substances, growth controls were carried out on the medium without the active agents.

To make a comparison, *C. albicans* ($n = 8$) isolates were used. These strains originated from mucous membrane swab samples. Precultivation was done on Sabouraud's 4% dextrose agar; susceptibility testing was performed on D.S.T. For agar dilution, yeast cell densities of 10^6 and 10^8 cfu/ml were used.

Both *Staphylococcus* (*S.*) *aureus* and coagulase-negative staphylococci ($n = 8$) served as reference strains with known *in vitro* susceptibility against the tested lipid components. Precultivation was done on Columbia blood agar (Heipha, Heidelberg, Germany), susceptibility testing also on D.S.T. agar with 10^6 and 10^8 cfu/ml.

The Mann–Whitney U-test was used for determination of significance.

RESULTS

M. furfur growth inhibition *in vitro* could be achieved only at extremely high concentrations of the PS. The lowest level of MIC values had PS base. The mean MIC value of 6,250 µg/ml corresponded to 0.63% content of PS in the agar (Table I). Thus, for the different PS salts – lactic acid salt, HCl and glycolic acid salt – even higher MIC values were needed; viz. 35,714 µg/ml (3.57%), 28,571 µg/ml (2.86%), and 46,429 µg/ml (4.64%).

For *Malassezia*, there were highly significant differences between MIC values of PS base and each of the PS salts ($p < 0.001$), but between different PS salts, there was no significant difference.

Growth of *C. albicans* strains was inhibited at PS concentrations between 152 ± 17 (mean ± SE) and 269 ± 41 µg/ml (cell density 10^6/ml). The glycolic acid salt was again the most active PS derivative (Table I). A highly significant difference between MIC values of PS base and PS lactic acid salt was found ($p < 0.0001$) when yeast cell density was 10^8/ml. However, for both 10^6/ml and 10^8/ml no significant difference was detected in case of *C. albicans* between the other lipid formulations.

PS were able to inhibit growth of the tested Gram-positive bacteria *S. aureus* and coagulase-negative staphylococci (CNS) *in vitro*. MIC values as they are shown in Table I were between 188 ± 0 and 515 ± 92 µg/ml. PS glycolic acid salt exhibited the lowest MIC value according to Table I (cell density of staphylococci 10^6/ml). MIC values of PS base and every other PS salt were nearly similar for *S. aureus* and CNS.

DISCUSSION

Sphingoid bases, e.g. PS, sphingosines and sphinganines are present in the stratum corneum in their free form and as constituents of ceramides, the most abundant lipids in the stratum corneum (10). The free form may be formed out of ceramides by enzymes – ceramidases – which catalyse the reaction to free sphingosines and free fatty acids (11). Depending on the equilibrium of this reaction, a fraction of the sphingoid bases will be in its free form. According to the literature, this is generally about 10% (12). The molar ratio between free PS, sphingosine and sphinganine in the stratum corneum – is 5.6:2.4:1 (corresponding to 2.8, 1.2, and 0.5 nmol/mg), which is a good reflection of the ratio of the different ceramides (based on their sphingoid base) in the skin (13).

The structural formula of the PS is shown in Fig. 1. Common synonyms of PS are (2S, 3S, 4R)-2-Amino-1,3,4-octadecanoetriol and 4D-Hydroxysphinganine.

Free sphingoid bases are stereo active compounds. In the skin they are only present in the D-erythro form. It is important to note that in structure-function studies it

Table I. *Minimum inhibitory concentrations of different phytosphingosines against Malassezia furfur, Candida albicans and staphylococci (mean ± SE)*

Microorganism	Cell density [cfu]	Phytosphingosine (Base)	Phytosphingosine lactic acid	Phytosphingosine HCl	Phytosphingosine/ glycolic acid salt
		Minimum inhibitory concentration [μg/ml]			
M. furfur ($n = 7$)	10^8/ml	6250 ± 0	35 714 ± 4724	28 571 ± 3341	46 429 ± 3 341
C. albicans	10^6/ml	176 ± 33	270 ± 41	211 ± 23	152 ± 17
($n = 8$)	10^8/ml	469 ± 61	750 ± 0	609 ± 141	773 ± 125
S. aureus/CNS	10^6/ml	375 ± 87	515 ± 92	305 ± 34	188 ± 0
($n = 8$)	10^8/ml	562 ± 94	867 ± 197	844 ± 207	961 ± 211

cfu = colony forming units; CNS = coagulase-negative staphylococci.

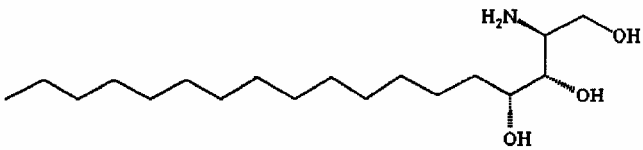

Fig. 1. Structural formula of phytosphingosine.

has been shown that differences in performance exist between the different stereoisomers of the sphingoid bases; the natural D-erythro form seems to be the most active isomer (14, 15).

Free sphingoid bases are known to mediate a wide variety of activities. For example, they are able to inhibit protein kinase C, and thus play a pivotal role in the regulation of cell growth. The action of free sphingoid bases seems to be an important factor in the modulation of epidermal cell proliferation in order to balance the rate at which cells are lost from the skin surface (15). Recently, Bektas et al. (16) demonstrated that synthetic ceramide analogues, e.g. N-thioacetylsphingosine, are able to induce apoptosis in human keratinocyte cell line HaCaT.

Furthermore, free sphingoid bases can stimulate the biosynthesis of ceramides in the skin. They act also as an anti-inflammatory, anti-oxidant, and antimicrobial agent (15, 17–19).

Bibel et al. (20) examined the *in vivo* effect of sphinganines (200 μg/cm^2) on the skin of human volunteers, first as a preventive antiseptic substance against subsequently applied *S. aureus* and *C. albicans*, and second as a restorative antiseptic agent against the previously expanded normal skin flora. In addition, sphingolipids were used for therapy of experimental *C. albicans* and *Trichophyton mentagrophytes* infection of guinea pigs. The authors were able to show that by application of 200 μg/cm^2 of sphinganine in ethanol, an up to three-log reduction in the population of target microorganisms was obtained, compared with vehicle and untreated controls. In addition, the daily application of sphingosine as 1.5% ethanol-petrolatum ointment was able to diminish inflammation slightly in dermatophyte-infected guinea pigs despite the fact that the animals remained culture positive over the 3 weeks' sampling period. In the *Candida* infections, the yeasts were eliminated in 75% of animals by the fourth day of therapy. They conclude, that these results further support simple sphingolipids as important antimicrobial agents of the cutaneous barrier and point toward a new biochemical approach in treating infectious disease.

Previously, it was shown that sphinganine and sphingosine kill staphylococci partly by damaging their cell wall (19). It could be demonstrated that PS at concentrations between 0.10 and 0.40 g/l are required for complete growth inhibition of different bacterial microorganisms, such as *S. aureus*, *Corynebacterium xerosis*, *Micrococcus luteus*, *Propionibacterium acnes*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Gram-positive bacteria proved to be more sensitive to PS than Gram-negative bacteria. This is in accordance with the MIC values of *S. aureus* and CNS demonstrated here, which were found to be in a range from 188 to 515 μg/ml. Furthermore, PS base and the different salts of this lipid formulation showed similar *in vitro* activity against the *C. albicans* strains investigated.

Recently, several studies could demonstrate *in vitro* activities of antifungals, e.g. ketoconazole, econazole, miconazole, but also of antiseborrhoeic, antipsoriatic agents and even *Melaleuca alternifolia* (tea tree) oil against *Malassezia* species (8, 21–23). *Malassezia* is the only fungus belonging to the physiological skin flora. Besides, this lipophilic yeast is the cause of common skin diseases, e.g. pityriasis versicolor, *Malassezia* folliculitis, and seborrhoeic dermatitis.

Our *in vitro* susceptibility testing of *Malassezia* led to the surprising result that growth of the investigated yeast strains was not inhibited at concentrations comparable to those required for *S. aureus*, CNS, and *Candida*. Obviously, *M. furfur* was almost unaffected by PS. Non-physiologic high concentrations of the different PS salts were necessary for growth inhibition. The only exception was PS base with MIC of 0.63%. This concentration is about 10 times higher than the MIC for the non-lipophilic microorganisms tested. Interestingly, lower concentrations of the lipid formulation led to an

observable growth stimulation of *Malassezia*. A possible explanation may be the dependence of the growth of *Malassezia* from lipids, in particular shorter chain fatty acids (C14–C18), commonly found in skin regions that are rich in sebaceous glands (24). Under *in vitro* conditions, olive oil and Tween contain these essential lipids allowing growth of the lipophilic fungus *Malassezia* (25). PS itself may serve as lipid source for *Malassezia*.

The presence of antimicrobial compounds such as PS and peptides in the epidermis leads to the hypothesis that the upper layers of the skin of both humans and animals contain a defence system that is preserved over evolution. The future use of these compounds for therapeutic purposes in common skin disorders seems to be possible.

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