

Long-term reduction in local inflammation by a lipid raft molecule in atopic dermatitis

S. Dölle¹, D. Hoser¹, C. Rasche¹, C. Loddenkemper², M. Maurer¹, T. Zuberbier¹ & M. Worm¹

¹Charité – Universitätsmedizin Berlin, Department of Dermatology and Allergology, Berlin; ²Charité – Universitätsmedizin Berlin, Institute of Pathology/Research Center ImmunoSciences (RCIS), Berlin, Germany

To cite this article: Dölle S, Hoser D, Rasche C, Loddenkemper C, Maurer M, Zuberbier T, Worm M. Long-term reduction in local inflammation by a lipid raft molecule in atopic dermatitis. *Allergy* 2010; DOI: 10.1111/j.1398-9995.2010.02341.x.

Keywords

atopic dermatitis; FoxP3; miltefosine; regulatory T cell; thermographic imaging

Correspondence

Prof. Dr. med. Margitta Worm
Charité – Universitätsmedizin Berlin
Department of Dermatology and Allergology
Charitéplatz 1
D – 10117 Berlin, Germany.
Tel.: +49 30 450 518 105
Fax: +49 30 450 518 931
E-mail: margitta.worm@charite.de

Accepted for publication 15 January 2010

DOI:10.1111/j.1398-9995.2010.02341.x

Edited by: Hans-Uwe Simon

Abstract

Background: The complex pathogenesis of atopic dermatitis (AD) is guided by cell surface receptor-mediated signal transduction regulated in lipid rafts. Miltefosine is a raft-modulating molecule targeting cell membranes. With this controlled clinical study, the clinical and immunomodulatory efficacy of miltefosine was investigated in patients with AD in comparison with a topical corticosteroid treatment.

Methods: Sixteen patients with AD were treated topically with miltefosine and hydrocortisone localized on representative AD target lesions for 3 weeks. To assess the clinical efficacy, the three item severity (TIS) score was evaluated before, during and after treatment as well as after 4-week-follow-up period. To study the anti-inflammatory effect of miltefosine on the cellular T cell pattern, skin biopsies were analysed before and after treatment.

Results: The TIS score dropped in both groups significantly after treatment. A carry-over effect was exclusively seen for miltefosine after discontinuing the treatment. These findings were substantiated by thermographic imaging with a significant decrease in the maximum temperature ($T_{\max}$) after miltefosine application ($P = 0.034$, $\Delta T_{\max} = 1.7^{\circ}\text{C}$ [2.1–3.9]). Immunohistochemically, a reduction in lesional CD4⁺-infiltrating T cells was observed in both treatments. Moreover, increased FoxP3⁺ cells were present in the skin after miltefosine treatment (before 5.4% [1.9–9.8], after 6.2% [3.5–9.5]).

Conclusion: We demonstrate that miltefosine is locally active in patients with AD and led to a sustained clinical improvement in local skin inflammation. Moreover, the increased frequency of FoxP3⁺ cells in the skin of patients with AD suggests its immunomodulatory properties.

The development of new therapeutic approaches for atopic dermatitis (AD) aims to target key molecules or specific pathways of the allergic inflammatory response (1–3). Current effort focuses on the use of small molecules as novel therapeutic alternatives for AD.

Many cell surface receptor-mediated signal transduction processes are involved in the pathogenesis of AD and are regulated in lipid rafts in the cell membrane including the immunoglobulin E (IgE)-receptor (FcεRI)-dependent mechanisms (4). Lipid rafts are phospholipid- and cholesterol-enriched domains in the cell membranes, which together with raft proteins form localized microdomains. The specific compositions and fluidity of these lipid rafts facilitate the effective interaction between transmembrane molecules and receptors, such

as with FcεRI (5). Therefore, targeting the lipid raft domains of human effector cells like mast cells, but also T cells and keratinocytes offers a novel therapeutic strategy for immune modulation.

Miltefosine (hexadecylphosphocholine) is a lipid raft-modulating agent by interfering cell membrane signal transduction pathways (6), like the phosphatidylinositol 3-kinase (PI3K)-Akt/protein kinase B (PKB) survival pathway (reviewed in (7)). Miltefosine (molecular weight: 408 D) is chemically similar to cell membrane phospholipids (Fig. 1) and accumulates in lipid rafts (8).

Like other alkylphosphocholines, miltefosine was originally developed as anti-tumoral agent (9) and has been approved for the topical treatment of skin metastases from breast

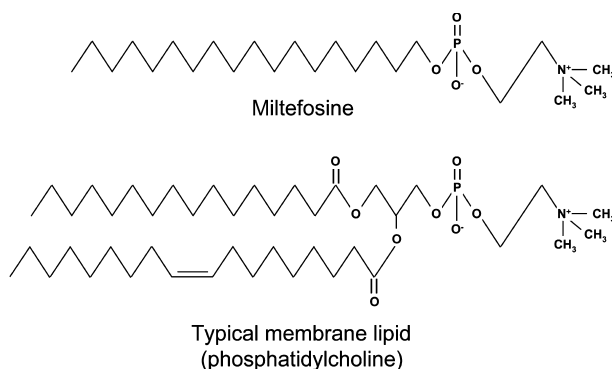


Figure 1 Chemical structure of miltefosine in comparison with typical membrane lipid (phosphatidylcholine).

cancer. The mechanism of action differs from classical chemotherapeutic drugs as miltefosine does not target the DNA (7). Its accumulation in the cell membrane causes a disturbance of the physiological phospholipid metabolism (8). Consequentially, it inhibits proliferation, but also induces differentiation (10) and apoptosis in a variety of tumours without being toxic for non-cancer cells (6). Miltefosine is the first effective oral agent available for treating visceral leishmaniasis (11) and has also been shown to be active in cutaneous leishmaniasis (12). In leishmaniasis, a deviated Th1/Th2 immune response is pathophysiologically relevant (13), and it has been shown that miltefosine acts on T cells (14). However, miltefosine retains its immunomodulatory properties in T-cell-deficient mice (15), suggesting a broader mode of action. Accordingly, miltefosine inhibits mast-cell-dependent immune responses (16).

Based on these observations and considering the need of novel local immunomodulatory approaches, we initiated a controlled prospective clinical study evaluating the clinical efficacy of topical miltefosine in comparison with a topical corticosteroid in patients with AD.

Material and methods

Patients

The trial protocol was approved by the ethic committee of Berlin and the competent authority. The patients were recruited at the Allergy-Centre-Charité (2007 and 2008), and written informed consent was obtained from each participant.

Patients suffering from AD aged ≥ 18 with two comparable skin lesions were enrolled into the clinical study. AD was defined according to the diagnostic criteria of Hanifin and Rajka (17). The main exclusion criteria were other dermatological inflammatory diseases, other chronic or acute illnesses requiring systemic treatment including immunodeficiency, systemically used immunosuppressive drugs including corticosteroids and immunomodulators, pregnancy and lactation.

Sixteen patients were enrolled into the study and completed the entire treatment (Fig. 2). Twelve patients gave

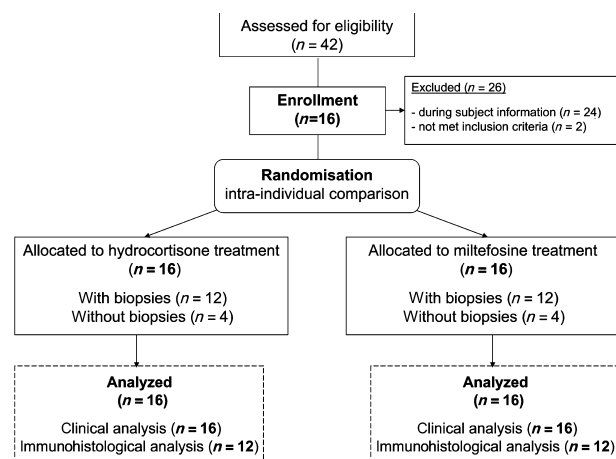


Figure 2 Study flow chart according to the CONSORT Statement of David Moher, Lancet 2001.

Table 1 Baseline characteristics of study population

Variable	Patients (n = 16)
Sex (female/male)	11/5
Age (years)	29 [18–58]
Height (cm)	175 [155–193]
Weight (kg)	71 [57–110]
Ethical origin	15 Caucasian/1 Asian
SCORAD (points)	51 [34–69]
<25 (mild)	n = 0 (0%)
≥ 25 and <50 (moderate)	n = 6 (37%)
≥ 50 (severe)	n = 10 (63%)
TIS score (points)	6 [5–7]
5 points	n = 7 (44%)
6 points	n = 7 (44%)
7 points	n = 2 (12%)

*TIS, three item severity; SCORAD, severity scoring of atopic dermatitis; values in median [minimum–maximum].

additionally informed consent for skin biopsies. Patients' characteristics are depicted in Table 1. All patients had moderate to severe AD as determined by means of SCORAD (severity scoring of atopic dermatitis) score (18), which was monitored over the study period. Laboratory examinations included full blood count, serum creatinine and liver parameters as well as calcium, sodium and potassium.

Treatment

Patients were treated for 3 weeks with topical application of 6% miltefosine solution (Milte[®], Baxter Oncology GmbH, Halle, Germany) and 1% hydrocortisone solution (Hydrogalen[®], GALENpharma GmbH, Kiel, Germany). Both vehicles were based on an alcoholic solution (Milte[®]: 3-propyloxy-propyleneglycol, 3-hexyloxypropyleneglycol, 3-nonyloxypropyleneglycol, citric acid, sodium hydroxide,

water; Hydrogalen®: 2-propanol, glycerol, propylene glycol, water). The study medications were randomly assigned as solution A and B, and each patient used two drops of the respective solution on the corresponding target lesion. The application scheme started with once daily the first week, and twice daily the second and third treatment week.

Clinical assessment

The primary efficacy parameter was the local clinical response to miltefosine assessed by the three item severity (TIS) score (firstly described by (19)). Briefly, the TIS score is the sum of three intensity items (erythema, oedema/papulation, excoriation) scored on a 4-point-scale from 0 to 3 (0 = none, 1 = mild, 2 = moderate, 3 = severe) with a maximum of 9 points. The TIS score of 2 target lesions (10 cm²) was determined before (day 1/visit 1), during (day 7/visit 2 and day 14/visit 3) and after (day 21/visit 4) as well as 4 weeks after finishing the treatment (follow-up) by the same dermatologist. All assessed lesions had a TIS score between ≥ 5 and ≤ 7 points before treatment.

The thermographic imaging (FLIR systems ThermoCAMTM S60, Frankfurt, Germany) was carried out in a room with only cold light sources. The camera was set at a distance of 30 cm from the target lesion and focused automatically. The pictures were taken before, during and after the treatment. The maximum temperature ($T_{\max}$) of the target lesion was analysed.

The transepidermal water loss (TEWL) was measured after 15 min rest with Tewameter® TM 300 (Courage-Khazaka, Cologne, Germany) before, during and after the treatment under standardized conditions regarding temperature and relative humidity.

Immunohistochemistry

Pre- and post-treatment 4 mm skin punch biopsies from both treatment areas were embedded and stored at -80°C . Skin sections of 4 μm were stained for CD4⁺ T cells by the streptavidin/biotin complex method using a monoclonal antibody to CD4 (clone MT310, Dako, Hamburg, Germany) and the alkaline phosphatase/red detection kit (Dako). The cells were counted in three consecutive 200 $\times$ 300- μm -fields at 100 $\times$ magnification, and the averages were calculated.

For measurements of the epidermal thickness, the 4 μm skin sections were stained with hematoxylin (Papanicolaou's solution, Merck, Darmstadt, Germany) and eosin (Eosin-Phloxin-solution, Dr. K. Hollborn&Söhne, Leipzig, Germany). Measurements were taken using the Axiovision measuring-tools (Zeiss, Berlin, Germany) at 100 $\times$ magnification.

For immunostaining of FoxP3 expressing cells (FoxP3⁺ cells) and the proliferation marker Ki-67, the 4 μm frozen sections were fixed in buffered formalin and subjected to a heat-induced epitope retrieval step before incubation with antibodies. Sections were immersed in sodium citrate buffer solutions at pH 6.0 and heated in a high-pressure cooker. The slides were rinsed in cool running water, washed

in Tris-buffered saline (pH 7.4) and incubated with the respective primary antibody. The primary antibodies included monoclonal mouse anti-Ki-67 (MIB-1, Dako) or rat anti-FoxP3 (PCH101, eBioscience, San Diego, USA). For detection, the streptavidin-AP kit (K5005, Dako) was used either alone or following biotinylated rabbit anti-rat (Dako) secondary antibody. Negative controls were performed by omitting the primary antibody. The cells were counted in three consecutive slides at 100 $\times$ (FoxP3) and 200 $\times$ magnification (Ki-67).

Statistical analysis

SPSS statistics 17.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analysis. All data are expressed as median [minimum–maximum]. Statistical analyses were performed with non-parametrical tests, either by the Mann–Whitney test (unpaired data) or by the Wilcoxon test (paired data). A P -value < 0.05 was considered statistically significant.

Results

Topical miltefosine treatment reduces local inflammation

To investigate clinical efficacy of topically applied miltefosine in AD, we analysed the TIS score before and after double-blinded topical anti-inflammatory treatment. The overall median TIS score of the lesions from all patients was 6 points [5–7] (Fig. 3A) before treatment started. All patients showed an improvement of 37% [17–100] in local skin inflammation of the miltefosine-treated lesions during the 3-week-therapy. Ten of 16 patients reached the primary outcome parameter defined as a decline of the TIS score > 1.5 points. Interestingly, the intensity of the miltefosine-treated lesions further declined by 17% [–50–67] 4 weeks after discontinuing the treatment (follow-up visit). The comparison of both treatments revealed a more pronounced decline of the TIS score in the hydrocortisone-treated lesions to a median of 2 points [1–6] (equal 73% [33–100]). However, this effect was not stable after discontinuing the hydrocortisone treatment, and a significant re-increase in the TIS score was determined ($P = 0.03$).

To substantiate our clinical observations, we examined the maximum temperature ($T_{\max}$) of the lesions measured by thermographic imaging and the TEWL. Thermographic imaging is a novel objective tool in the assessment of changes in inflammatory mechanisms (20, 21). We observed a significant median reduction for the $T_{\max}$ in the miltefosine-treated lesions ($P = 0.034$, $\Delta T_{\max} = 1.7^{\circ}\text{C}$ [2.1–3.9]; Fig. 3B). No significant changes of $T_{\max}$ were detected in the hydrocortisone-treated lesions ($\Delta T_{\max} = 0.9^{\circ}\text{C}$ [2.4–7.9]).

Consistent with the data from Jensen et al. (22, 23), we detected increased TEWL values before both, miltefosine and hydrocortisone treatment, in our patients suffering from AD. Over the 3-week-treatment phase, we determined a significant reduction in TEWL values in both groups (Fig. 3C). However, the comparison between miltefosine and hydrocortisone revealed no differences.

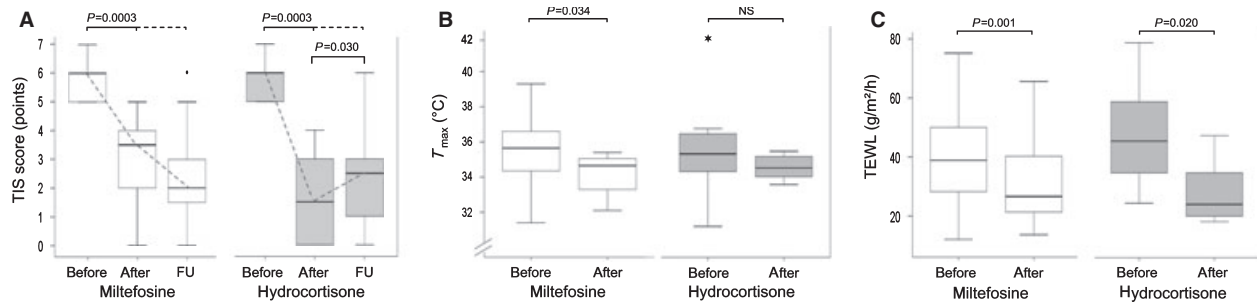


Figure 3 Clinical parameters upon miltefosine and hydrocortisone treatment. (A) Before, after and at follow-up (FU) the three item severity (TIS) score was assessed in the target lesion. (B and C) As surrogate parameters of inflammation thermographic measurements, depicted as the maximum temperature (T_{max}) of the

target lesions (B), and transepidermal water loss (TEWL) (C) were measured before and after the treatment in addition. $N = 16$, outliers are shown as black dots and the extreme values as black asterisks, NS – not significant.

The SCORAD score was monitored throughout the study period as well to assess the overall disease activity. A low, but significant reduction in the SCORAD score (before 51.2 [34.3–68.8] points, after 39.7 [19.7–67.0] points, $P = 0.005$) was observed over the 3-week-treatment.

Miltefosine modulates the skin-infiltrating T cell subsets

To study the effects of the topical anti-inflammatory treatment on the cellular T cell pattern in patients with AD, we analysed skin biopsies from 12 patients before and after treatment with miltefosine and hydrocortisone.

The infiltrates of $CD4^+$ T cells were strongly present in the upper dermis of all lesions before treatment. Representative examples of the immunohistochemical staining of $CD4^+$ T cell infiltrations are shown in Figure 4A. Hydrocortisone-treated lesions showed a significant reduction in $CD4^+$ T cells, whereas miltefosine-treated lesions had only slightly reduced $CD4^+$ T cell infiltrations (Fig. 4B).

Miltefosine increased the frequency of FoxP3⁺ cells in the skin

To investigate the immunomodulatory effect of miltefosine on regulatory T cells, we stained FoxP3 in the skin sections before and after treatment (Fig. 4C). The proportions of FoxP3⁺ cells were comparable between the target lesions of every patient before treatment. The median proportion of FoxP3⁺ cells of $CD4^+$ cells was 5.4% [1.9–9.8] and 5.0% [2.2–10.6] in the miltefosine- and hydrocortisone-treated lesions, respectively. An increase in the FoxP3⁺ cell fraction was detected after miltefosine treatment (6.2% [3.5–9.5], Fig 4D). In contrast, the hydrocortisone treatment led to a reduction in FoxP3⁺ cells (3.5% [1.9–8.3]).

Miltefosine caused no epidermal atrophy

Increased epidermal thickness may be associated with increased epidermal proliferation and has been previously

described in patients with AD (22). Herein, we analysed the epidermal thickness and proliferating cells (Ki-67⁺) in the basal layer. The epidermal thickness was comparable between both target lesions before treatment. The medians of epidermal thickness with 205.74 μ m (lesions allocated to miltefosine) and 205.65 μ m (lesions allocated to hydrocortisone) were increased compared to skin of healthy controls (23). Hydrocortisone treatment led to a significant reduction in epidermal thickness (Fig. 5A) accompanied by reduced individual cell size (Fig. 5D), which represents an early marker for atrophy (24). In contrast, the individual cell size was unaffected in the miltefosine-treated lesions (Fig. 5C).

A similar picture was seen regarding the proliferation rate measured by the presence of Ki-67⁺ cells in the basal layer of the epidermis (Fig. 5B). Miltefosine treatment decreased the proportion of Ki-67⁺ cells in only 6/12 lesions without being statistically significant (Δ proportion of Ki-67⁺ cells = 2.1% [−170.0–63.0]). In contrast, 10/12 lesions treated with hydrocortisone exhibited a significant reduction in Ki-67⁺ cells ($P = 0.024$, Δ proportion of Ki-67⁺ cells = 54.2% [−81.3–91.4]).

Topical miltefosine treatment is well tolerated

The application of miltefosine and hydrocortisone over 3 weeks was well tolerated. Neither any systemic adverse event (AE) occurred nor any AE caused a pretermination of the intervention. However, local skin-related AEs linked to the topical treatments occurred during the study period. Ten of 16 patients (63%) and 7 of 16 patients (44%) of the miltefosine and hydrocortisone treatment, respectively, reported on local AEs like pruritus, burning, tingling and dry skin. Skin-related AEs occurred more often in the miltefosine-treated lesions. In particular, symptoms of dry skin were exclusively observed in the miltefosine-treated lesions. However, these local AEs were usually temporary. All safety laboratory values and vital signs were in a normal range before and after the treatment.

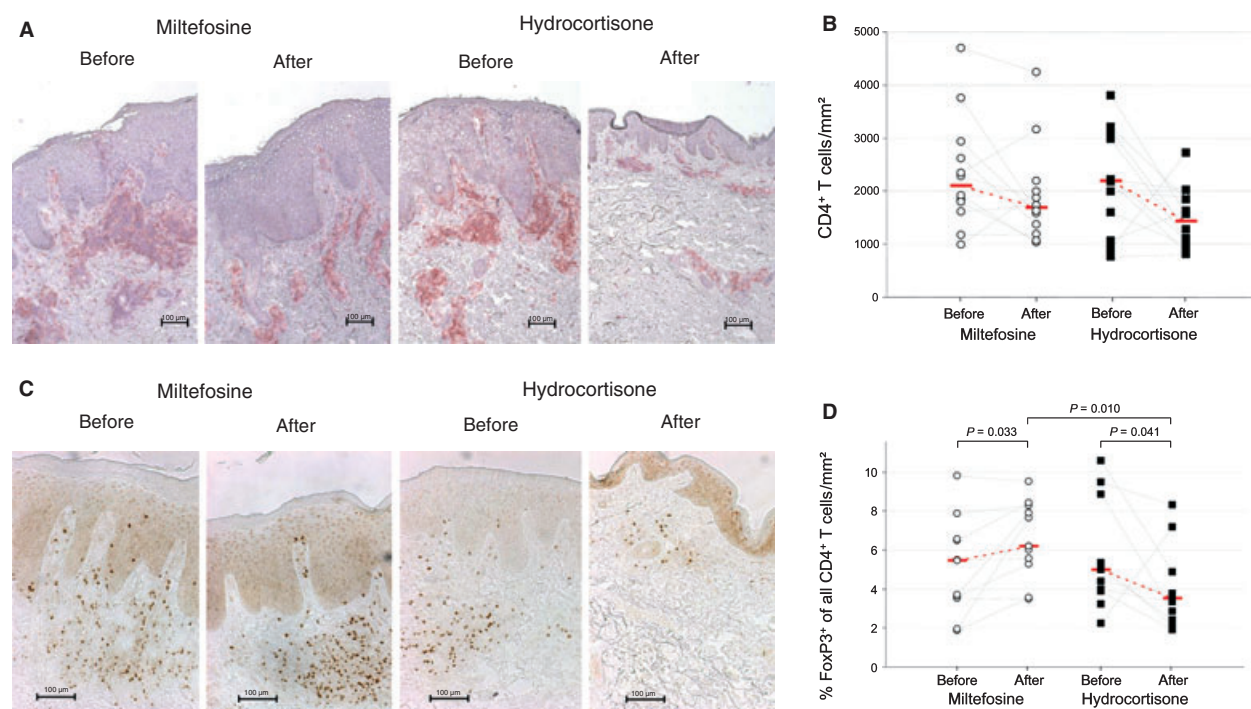


Figure 4 Frequencies of skin-infiltrating cell subsets. (A–B) Reduction in infiltrating CD4⁺ T cells upon treatment; (A) Representative immunohistochemical staining of CD4⁺ T cell (red) before and after a 3-week-treatment; (B) Individual analysis of CD4⁺ T cells before and after the treatment with miltefosine (white circles) and hydrocortisone (black squares), $n = 12$, the median is shown as a red bar,

NS – not significant; (C–D) Increased frequencies of FoxP3⁺ cells by miltefosine, but not hydrocortisone treatment; (C) Representative immunohistochemical FoxP3 staining (brown); (D) differential expression of FoxP3⁺ cells by miltefosine (white circles) and hydrocortisone (black squares), $n = 11$, the median is shown as a red bar.

Discussion

In the present study, we demonstrate that miltefosine, a small lipid raft-modulating molecule, has anti-inflammatory effects in patients with AD. Miltefosine led to a clinical improvement in local skin inflammation with a carry-over effect and was not atrophogenic. Moreover, the increased frequency of FoxP3⁺ cells indicated its immunomodulatory action in a topical application. The use of novel, small molecules might expand the established topical treatment of AD by actively changing the immune machinery with the aim of long-term modulation.

T helper cells play a crucial role in the pathogenesis of AD (25). Herein, the short-term treatment with miltefosine failed to significantly reduce the lesional CD4⁺ T cell population but increased numbers of FoxP3⁺ cells were detected. In contrast, the number of FoxP3⁺ cells significantly decreased in the hydrocortisone-treated lesions. These findings raise several questions, which include in particular the questions whether these cells result from a better survival or an increased local proliferation because of changes of the local cytokine milieu or both, or even result from increased immigration of these cells into the skin. Those questions have been addressed by other authors previously (26–28), but remain to be elucidated. The immunomodulatory effect

of miltefosine seen in this study may be because of an enhanced frequency of regulatory T cells (Tregs) in the skin. Miltefosine was previously shown to induce IL-2 receptor (CD25) expression on T cells (29). Furthermore, genetic evidence that Tregs are essential in regulating skin inflammation is provided by the loss-of-function mutation in the *foxp3* gene, which is accompanied by severe generalized eczema (30). Accordingly, lesional FoxP3⁺ Tregs express the cutaneous lymphocyte-associated antigen (26). But as the expression of FoxP3 is not exclusive for Tregs and may be transiently up-regulated in conventional T cells after activation as well (31), a clear differentiation is necessary in future studies.

In this study, we identified a novel therapeutic concept, which resulted in a clinical benefit, which was associated with an enhancement of FoxP3⁺ cells in the skin, unlike established therapies as UVA1 (32), topical tacrolimus or hydrocortisone (33). The three item severity (TIS) score, as our primary clinical parameter, was significantly reduced upon the short-term treatment with miltefosine and showed a disease modifying effect over the 4-week-follow-up period. Although the efficacy of miltefosine was less compared to hydrocortisone, no relapse in the lesions was observed upon discontinuing the treatment, indicating a beneficial biological profile compared to hydrocortisone. The phenomenon of

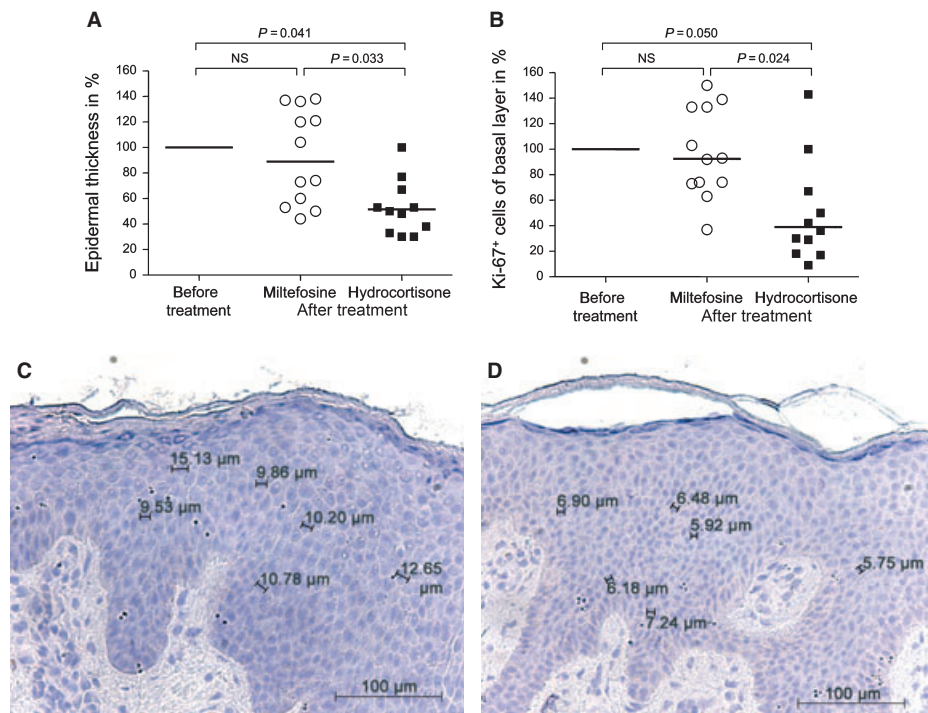


Figure 5 Analysis of epidermal proliferation and atrophogenic effect. (A) Changes in epidermal thickness because of miltefosine treatment (white circles) and epidermal atrophy because of hydrocortisone treatment (black squares); (B) Decline in cell proliferation depicted as number of Ki-67⁺ cells in the basal layer after milte-

fosine (white circles) and hydrocortisone treatment (black squares); $n = 12$, the median is shown as a black bar, NS – not significant; (C and D) Representative H&E stainings (C) after miltefosine treatment without visible reduction in cell size, (D) after hydrocortisone treatment with visibly reduced cell size.

relapse is well known for corticosteroids and limits its therapeutic use in the long term.

The disease activity was assessed as well. A low, but significant reduction in the SCORAD score was observed throughout the study, which is probably because of the well-known doctor-care-effect.

Additionally, our clinical findings were supported by thermographic measurements, which allow to quantify changes in skin surface temperature caused by inflammatory actions (20) and by the TEWL, which correlates to the epidermal barrier function. Here, we determined a significant decrease in maximum temperature ($T_{\max}$) in the miltefosine-treated lesions indicating less inflammatory activity, which paralleled by a decreased TIS score. However, no remarkable changes were observed in the hydrocortisone-treated lesions. Consistently, we detected decreased TEWL values after both treatments. A limitation of our study is that patients were allowed to use their regular anti-inflammatory treatment for the remaining eczema on demand. However, only a subgroup of seven patients used this possibility. To exclude indirect effects of such anti-inflammatory treatments on the study results, it was prohibited to use corticosteroids in proximity of the target lesions (20 cm distance).

In this study, we show that miltefosine treatment did not significantly alter the epidermal proliferation in contrast to hydrocortisone. As increased proliferation is not only

reflecting inflammation, which was more or less reduced in both groups, but also essential in physiologic skin turn over (22), the reduced proliferation rate down to >50% after hydrocortisone treatment indicates an atrophogenic effect. Skin atrophy is a well-known side-effect of topical corticosteroids (34), and atrophogenicity is characterized by a decrease in the cell size rather than cell numbers (24). In the present study, miltefosine treatment was not atrophogenic as the epidermal thickness and proliferation were not significantly altered. Hence, it might be possible that miltefosine restores the impaired epidermal barrier by normalizing the epidermal thickness without atrophogenic qualities.

Miltefosine was originally developed for the topical treatment of breast cancer metastasis and has been proven in this setting as a safe and well-tolerated drug (35). The circulating drug levels after topical application are below the detection limit in humans. The risk of systemic toxicity is acceptably low, and systemic adverse events (AEs) are not expected and indeed did not occur in this study. Patients suffering from AD and in particular those with severe and widespread eczema with a genetic concomitant skin barrier defect may get higher circulating drug levels and should be explored in future pharmacokinetic studies. Local pruritus and dry skin occurred more often during miltefosine treatment compared to hydrocortisone. These AEs may be contributed by the

high alcoholic content of the formulation (Milte[®]). However, by using an optimized formulation its value as an immunomodulatory substance in the treatment of AD may be achieved.

In summary, we demonstrate that miltefosine has anti-inflammatory effects in patients with AD. Miltefosine led to a sustained clinical improvement in local skin inflammation without atrophogenicity. This data is strengthened by the observation that inflammation-related parameters like $T_{\max}$ and TEWL were improved after miltefosine treatment as well. Moreover, the increased frequency of FoxP3⁺ cells in the skin suggests its potential immunomodulatory properties. However, the exact impact of FoxP3⁺ cells or Tregs on the clinical improvement observed in our miltefosine-treated patients needs to be clarified. Our data indicate the potential of miltefosine as novel topical immune modulator, which can be used for the treatment of AD, provided that

the AEs do not greatly increase when larger skin areas are treated. However, the data should be validated in a larger study cohort.

Acknowledgments

We thank JADO Technologies GmbH, Dresden, Saxony, Germany for the support of the clinical study and their helpful cooperation. We also thank Dr. G. Heine for critical discussion of the manuscript and Dr. H.-H. Lee for clinical assistance. The study was in part funded by the Deutsche Forschungsgesellschaft (DFG - SFB650 TP5 to MW).

Conflict of interest

All authors declare that there is no conflict of interest regarding the clinical study described in the manuscript.

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