The susceptibility of trichophyton rubrum to photodynamic treatment

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Chapter VIII

INVESTIGATION OF THE BEHAVIOUR OF THE CATIONIC PHOTOSENSITIZER 5,10,15-TRIS (4-METHYLPYRIDINIUM)-20-PHENYL-[21*H*,23*H*]-PORPHINE TRICHLORIDE AFTER ITS APPLICATION ON HEALTHY SKIN AND STRATUM CORNEUM INFECTED WITH THE DERMATOPHYTE *TRICHOPHYTON RUBRUM*

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Submitted

Trichophyton rubrum hyphae after photodynamic treatment with a lethal Sylsens B concentration, showing a complete loss of the fibre-mesh wall material

ABSTRACT

Dermatophytes cause superficial infections of keratinized tissues. Recently, we have demonstrated that the most frequently occurring dermatophyte, *Trichophyton rubrum*, could be destroyed with photodynamic treatment (PDT) with a 5,10,15-tris (4-methylpyridinium)-20-phenyl-[21*H*,23*H*]-porphine trichloride (Sylsens B) formulation with low ion content and pH 5.2. For a save application of Sylsens B in clinical PDT of dermatophytoses it is neither necessary nor desirable that Sylsens B penetrates the skin. To investigate whether this effective Sylsens B formulation could guarantee low Sylsens B penetration in healthy skin and skin infected with *T. rubrum*.

Sylsens B skin penetration studies were performed with dermatomed skin, human stratum corneum (SC), disrupted human SC by *T. rubrum* growth and human SC pre-treated with a detergent. The effective Sylsens B formulation (pH 5.2) was compared to a formulation in PBS (pH 7.4). Visualisation in dermatomed skin was performed with confocal scanning laser microscopy (CSLM).

There was no Sylsens B penetration in healthy skin at pH 7.4 or 5.2. Disruption of SC by preceding fungal growth caused Sylsens B to penetrate at pH 7.4, but not in case of our PDT formulation with pH 5.2, while chemically damaged SC caused Sylsens B penetration also at pH 5.2. CSLM investigations confirmed that in dermatomed skin Sylsens B did not reach viable epidermis and dermis.

The presence of *T. rubrum* on SC prevented Sylsens B to penetrate when using the effective PDT formulation (low ion content and pH 5.2). Therefore, this formulation may be safe for a future clinical PDT of dermatophytoses caused by *T. rubrum*.

INTRODUCTION

Dermatophytes are fungi that cause infections of superficial keratinized tissues (1,2). These infectious agents rarely invade deeper layers of the skin. The most commonly isolated dermatophyte with worldwide distribution is *Trichophyton rubrum* (3,4).

The present treatment strategies (topical and/or oral drug application or a combination of both) are not always curative (5,6). One of the reasons is that the current treatments inhibit mainly the metabolic active fungus (1,7-9), and leave the spores unaffected. Moreover, it has been reported that the dermatophyte *T. rubrum* can reduce the host's immune response rendering it more resistant to the currently used treatments (10).

Recently, we have demonstrated that a single photodynamic treatment (PDT) with the photosensitizer 5,10,15-tris(4-methylpyridinium)-20-phenyl-[21*H*,23*H*]-porphine trichloride (Sylsens B, Fig 8.1) applied to *T. rubrum* grown on human stratum corneum (SC) resulted in complete fungal kill (fungicidal effect) (11,12).

Photodynamic treatment refers to the use of light-activated agents called photosensitizers (13). Upon irradiation with light of an appropriate wavelength, photosensitizers can initiate a photochemical reaction resulting in the production of reactive oxygen species. This sequence of these events is known as photodynamic effect and it can cause the elimination of pathogens (14). The use of PDT for fungal infections is a new and promising approach (12,15). Effective PDT requires a selective binding of the photosensitizer to the target organism (14,16). In case of *T. rubrum,* our previous results showed that the fungicidal PDT effect was indeed achieved after the selective binding of the positively charged porphyrin Sylsens B to the negatively charged outer wall of fungal microconidia or hyphae. This binding could be accomplished with a Sylsens B formulation when low ion strength and a pH of 5.2 were used (12).

For a topical application of Sylsens B in patients with dermatophytosis it is neither necessary nor desirable that Sylsens B penetrates through the SC into the viable epidermis and dermis. A formulation that does not cause skin penetration of Sylsens B in neither healthy nor dermatophytosis infected skin is therefore to be preferred in future clinical experiments.

We therefore investigated the penetration behaviour of Sylsens B in healthy dermatomed skin, normal human SC, human SC infected with *T. rubrum* microconidia, and human SC pre-treated with a detergent. Reproducible fungal growth on SC was achieved by utilizing two fungal growth stages that corresponded to 3 and 5 days after the spore inoculation. The permeation studies were performed with Sylsens B in

two formulations: i) the previously successfully tested formulation containing a low molarity buffer of pH 5.2 (12) and ii) Sylsens B in phosphate buffered saline (PBS), at pH 7.4. Since it has been reported that sodium lauryl sulphate (SLS) reduces the barrier function of the SC (17), we also examined (as a positive control) the penetration of Sylsens B (at pH 5.2) through SC pre-treated with SLS.
In order to investigate whether Sylsens B could be visualized in SC and the viable skin, the dermatomed skin was cross-sectioned after the diffusion studies and visualized using confocal scanning laser microscopy (CSLM).

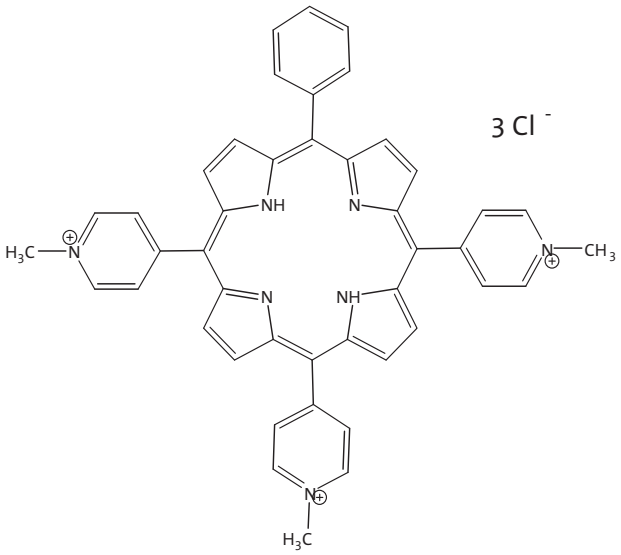

Figure 8.1. Chemical structure of the porphyrin photosensitizer 5,10,15-tris(4-methylpyridinium)-20-phenyl-[21*H*,23*H*]-porphine trichloride (Sylsens B).

MATERIALS AND METHODS

Materials

The fungus *T. rubrum* was purchased from the Centraalbureau voor Schimmelcultures (CBS, strain no: 304.60), Utrecht, The Netherlands. For the preparation of a microconidia suspension *T. rubrum* cultures were grown on Sabouraud Dextrose Agar (Sigma-Aldrich Chemie, Germany) at room temperature.
Sylsens B (mol wt: 769.16 g/mol) was synthesized by Buchem Holding BV (Lieren, The Netherlands). Its purity, determined with NMR was more than 99 %. Trypsine was

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obtained from Sigma (Zwijndrecht, The Netherlands), while all other chemicals were purchased from J.T.Baker (Deventer, The Netherlands).
Solutions of Sylsens B were made in either PBS (pH 7.4) or in a 5 mM citric acid/sodium citrate buffer (pH 5.2) and stored at 4°C for no longer than one week.

Preparation microconidia suspension

The protocol for obtaining a suspension of microconidia produced by *T. rubrum* grown on Sabouraud Dextrose Agar was based on a method described previously (11,18). The obtained microconidia suspensions (10– 40 x 10^6 colony forming units (cfu) /mL) were stored in liquid nitrogen for no longer than 6 months. Counting the number of cfu on Malt Extract Agar (MEA) dishes was used as a viability check.

Preparation of human dermatomed skin and SC

Abdominal or breast skin was obtained from a local hospital after cosmetic surgery. After removal of the fat tissue, the skin was cleaned with distilled water and dermatomed to a thickness of approximately 200 to 250 µm using a Padgett Electro Dermatome Model B (Kansas City, USA). For the preparation of the SC the dermatomed skin was incubated at the dermal side with a 0.1 % trypsin solution in phosphate buffered saline of pH 7.4 (4°C) overnight. After 1 hour at 37°C, the SC was removed manually. The obtained SC was dried in the air for 24 hours and kept under nitrogen over silicagel for no longer than 3 months.
For the penetration studies, circular sheets of 18 mm in diameter of dermatomed skin or SC were used. The SC was used either directly, after 24 hours pre-treatment with 1% SLS (followed by 5 washing steps to remove the SLS) or after infection with *T. rubrum*. For the latter purpose, the SC was placed in the central part of a polycarbonate membrane filter, 25 mm in diameter and a pore size of 2 µm (Omnilabo, Breda, The Netherlands) on a 3 cm culture dish (Greiner, Alphen aan den Rijn, The Netherlands) filled with 5 mL of Malt Extract Agar. A microconidia suspension was diluted to 175 cfu/ml and 20 µL inoculated in the centre of the SC. After the inoculation, the dish was placed in an incubator at 28°C for 3 or 5 days and the required number of fungal colonies checked microscopically prior to the penetration experiment. In this way, reproducible skin barrier damage could be obtained due to growth of 3 to 4 fungal colonies at 3 and 5 days after spore inoculation.

Penetration studies

In vitro penetration studies were performed using Teflon diffusion cells (see Fig. 8.2), designed and produced by the Department of Fine Mechanics of the University of Leiden. The diffusion area of the cells was 0.78 cm^2 and the volume of the acceptor chamber 0.98 cm^3.

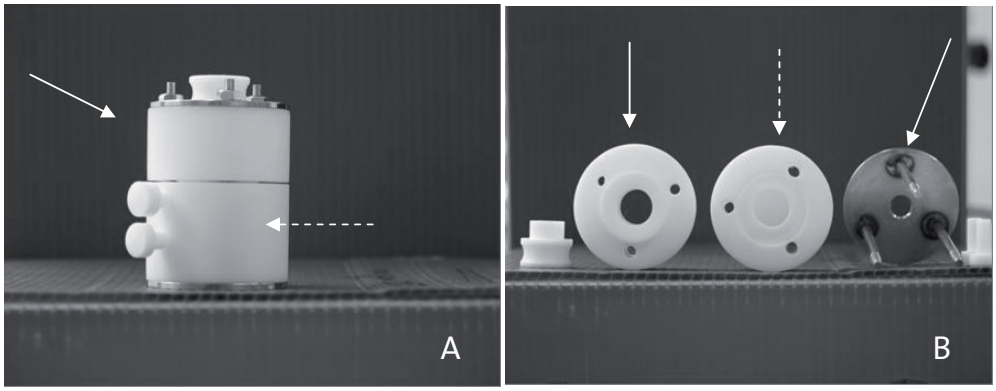

Figure 8.2. Photograph of the Teflon diffusion cell used for the penetration studies. The cell consists of an upper donor chamber (arrow in A and white left arrow in B) and a lower acceptor chamber (dashed arrow in A and B). The skin or SC is placed in the centre of the acceptor chamber that is subsequently filled with acceptor liquid. After filling the acceptor chamber and removal of air bubbles the chamber is closed. The donor chamber is mounted to the acceptor chamber, filled with donor liquid and finally the cell assembled with the screwing system as shown by the right arrow in B.

A circular piece of SC or dermatomed skin with a thickness of approximately 200 to 250 µm and a diameter of 18 mm was placed in the centre of the acceptor chamber. The acceptor chamber was filled either with PBS (pH 7.4) supplemented with 5 % ethanol or 5 mM citric acid/sodium citrate buffer (pH 5.2) with 5 % ethanol and the donor chamber with 300 µL Sylsens B solution of 80 and 160 µM (respectively 62 and 123 µg/ml). Controls contained a donor chamber filled with PBS (pH 7.4) or 5 mM citric acid citric acid/sodium citrate buffer (pH 5.2). The cells were placed for 24 hours (under occlusive conditions) in a water bath with a temperature of 32°C. Then before disassembling the cell the SC or dermatomed skin was washed with buffer and the acceptor volume was collected (syringe) and weighed. The concentration of Sylsens B in the acceptor phase was determined by measuring the fluorescence at 657 nm upon excitation at 424 nm (Perkin Elmer LS 50B luminescence spectrometer, Perkin Elmer Nederland BV). For every pH, a calibration curve was made to determine Sylsens B concentration in the acceptor phase. Each experiment contained 3 cells for each test

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condition and experiments were repeated at least 6 times using skin from different donors.

Confocal scanning laser microscopy

After the penetration experiments with dermatomed skin, the skin was cross-sectioned using a cutting device based on the device as described earlier (19) (illustrated in Fig. 8.3A). In addition to the device as described we included perplex holders to support the silicon sheets. The cross-sectioned skin was mounted in a sample holder positioning the cutting surface against the cover glass (Fig. 8.3B). The CSLM was carried out using a BioRad Radiance 2100 MP (BioRad, Hertfordshire UK) equipped with an Argon/HeNe laser. Samples were examined approximately 20 µm below the cutting surface using a 514 nm laser line with a Flaur 40x 1.30 oil immersion objective. Confocal images were collected (at least 10 for every condition) and digitized using Zeiss Lasersharp 2000 and the image-Pro 3Ds 5.1 software program. All images taken were averages of 10 scans.

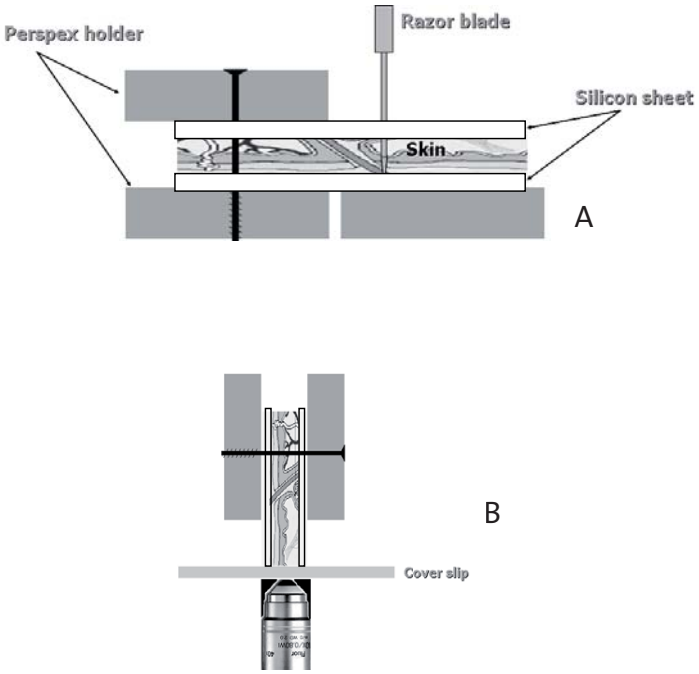

Figure 8.3. Diagram of the cutting device used to obtain a cross-section of the skin for CSLM imaging. After the penetration experiment, a square piece from the skin diffusion area, was pinched between two silicon sheets and fixed in perplex holders as shown in A. After cutting the skin (sandwiched between the silicon) perpendicular from dermis to SC, it was mounted in a sample holder as shown in B.

Statistical analysis

For statistical analysis the independent student-*t*-test was applied (GraphPad Prism 3.02) using critical level of significance of 0.05 (*P* values gives are 2-tailed).

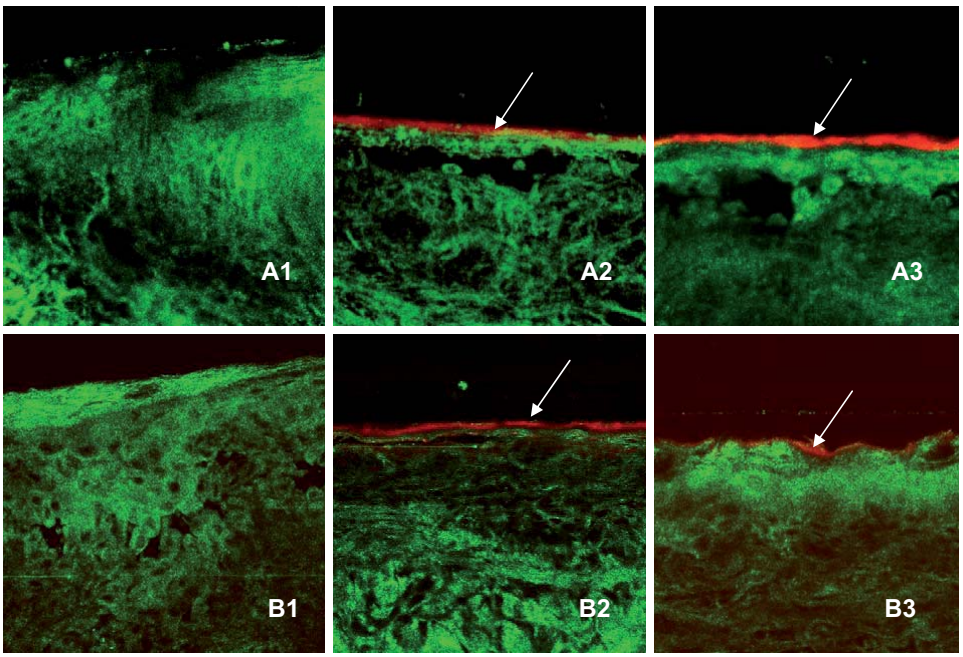

Figure 8.4. CSLM images of cross-sections of human skin after the skin penetration experiment at pH 7.4 (series A) and pH 5.2 (series B). PBS, pH 7.4 (A1), 80 µM Sylsens B in PBS (A2), 160 µM Sylsens B in PBS (A3), 5 mM citric acid, pH 5.2 (B1), 80 µM Sylsens B in 5 mM citric acid, pH 5.2 (B2) and 160 µM Sylsens B in 5 mM citric acid, pH 5.2 (B3). Arrows indicate the fluorescence of Sylsens B. Magnifications used: 400 X.

RESULTS

Sylsens B does not penetrate dermatomed human skin

When using dermatomed human skin we observed no penetration of Sylsens B even when we applied the highest concentration of this photosensitizer (160 µM) at both pH 7.4 and 5.2 (see Table 1). The results show that the fluorescence intensity measured in the acceptor chamber after 24 hours application of Sylsens B at pH 7.4 did not differ significantly from the control. At pH 5.2, similar results were obtained. In our CSLM experiments the absence of Sylsens B in the viable epidermis and dermis was evident (Fig. 8.4). The SC, the viable epidermis and the dermis are visible due to the green autofluorescence at 514 nm (see Fig. 8.4 A1 and B1 for 7.4 and 5.2 respectively). It

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could be noticed that at both pH, the orange fluorescence of Sylsens B was localized only on the surface and did not penetrate into the deeper skin layers.

Donor Chamber Sylsens B concentration (µg/ml)	*Acceptor Chamber Fluorescence[a] (AU)*	
	pH 7.4	*pH 5.2*
0	8.6 ± 0.7	11 ± 3
62	11 ± 2	11 ± 3
123	11 ± 3	12 ± 3

Table 8.1. *In vitro* penetration behavior of Sylsens B through human dermatomed skin at pH 7.4 (PBS) and 5.2 (5 mM citric acid/sodium citrate buffer).
[a] The accumulated amount of fluorescence (in AU) measured in the acceptor liquid after 24 hours at 657 nm upon excitation at 424 nm. Results as given mean ± standard deviations

A skin barrier damaged by fungal growth causes Sylsens B penetration at pH 7.4, but not at pH 5.2.

The influence of preceding fungal growth on the Sylsens B penetration through SC was investigated on SC sheets infected with *T. rubrum*. The sheets were used at two different time points after the spore inoculation. The results are provided in the Table 8.2 (pH 7.4) and Table 8.3 (pH 5.2).

Donor Chamber	*Acceptor Chamber*			
	3 days fungal growth		*5 days fungal growth*	
Test condition	*Fluorescence (AU)*	*[Sylsens B] (µg/ml)*	*Fluorescence (AU)*	*[Sylsens B] (µg/ml)*
PBS, pH 7.4 (no fungus)	8 ± 3	n.p	10 ± 1	n.p
Sylsens B (123 µg/ml) in PBS, pH 7.4 (no fungus)	12 ± 3	n.p	13 ± 3	n.p
PBS, pH 7.4 (fungus present)	9 ± 2	n.p	12 ± 2	n.p
Sylsens B (123 µg/ml) in PBS, pH 7.4 (fungus present)	10 ± 1	n.p	64 ± 12[a]	0.074 ± 0.024[a]

Table 8.2. *In vitro* penetration behavior of Sylsens B through SC infected with *T. rubrum* at pH 7.4.
Studies were performed at 3 and 5 days after the *T. rubrum* spore inoculation. The accumulated amount of fluorescence (in AU) measured in the acceptor liquid after 24 hours at 657 nm upon excitation at 424 nm. Results as given mean ± standard deviations.
[a] Significantly different compared to uninfected SC ($t(10) = 8.034$, $P = 0.0002$) and to the application of PBS ($t(10) = 8.310$, $P = 0.0002$).
n.p: no Sylsens B present.

The results obtained at pH 7.4 showed that there was no Sylsens B penetration through uninfected SC (control) and SC three days after microconidia inoculation. However, 5 days after inoculation, a considerable increase in fluorescence was detected in the acceptor fluid, which was significant different from uninfected SC. Also the application of Sylsens B to infected SC resulted in a significantly higher fluorescence in the accepter phase when compared to the application of the control PBS in the presence and absence of fungus growth.

Donor Chamber	***Acceptor Chamber***			
Test condition	**3 *days fungal growth***		**5 *days fungal growth***	
	Fluorescence (AU)	***[Sylsens B] (µg/ml)***	***Fluorescence (AU)***	***[Sylsens B] (µg/ml)***
Citric acid/sodium citrate, pH 5.2 (no fungus)	7 ± 3	n.p	6 ± 2	n.p
Sylsens B (123 µg/ml) in Citric acid/sodium citrate, pH 5.2 (no fungus)	10 ± 3	n.p	7.8 ± 0.7[a]	0.0021 ± 0.0008[a]
Citric acid/sodium citrate, pH 5.2 (fungus present)	6 ± 2	n.p	8 ± 2	n.p
Sylsens B (123 µg/ml) in Citric acid/sodium citrate, pH 5.2 (fungus present)	8 ± 2	n.p	12 ± 5	n.p

Table 8.3. *In vitro* penetration behavior of Sylsens B through SC infected with *T. rubrum* at pH 5.2.
Studies were performed at 3 and 5 days after the *T. rubrum* spore inoculation. The accumulated amount of fluorescence (in AU) measured in the acceptor liquid after 24 hours at 657 nm upon excitation at 424 nm. Results as given mean ± standard deviations.
[a] Significantly different ($t(10) = 2.482$, $P = 0.0324$) from the control with the calculated amount of Sylsens B penetrated (µg Sylsens B/ml acceptor liquid).
n.p: no Sylsens B present.

When pH 5.2 was used, similar results were obtained with the exception of the experiments using SC five days after spore inoculation. In this case, no penetration of Sylsens B was observed at pH 5.2 in contrast to the situation when the photosensitizer was dissolved in pH 7.4 (compare Table 8.2 to 8.3). Even when the highest concentration of Sylsens B was applied there was no statistically significant difference with controls. However, when the SC was pre-treated for 24 hours with 1% SLS, a significant amount of Sylsens B (applied at pH 5.2) was detected in the acceptor phase (see Table 8.4).

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Donor Chamber	*Acceptor Chamber*	
Test condition	*Fluorescence (AU)*	*[Sylsens B] (µg/ml)*
Citric acid/sodium citrate, pH 5.2 (normal SC)	7 ± 3	n.p
Sylsens B (123 µg/ml) in Citric acid/ sodium citrate, pH 5.2 (normal SC)	5 ± 1	n.p
Citric acid/sodium citrate, pH 5.2 (SC pre-treated with SLS)	5 ± 2	n.p
Sylsens B (123 µg/ml) in Citric acid/sodium citrate, pH 5.2 (SC pre-treated with SLS)	124 ± 109[a]	0.10 ± 0.08[a]

Table 8.4. *In vitro* penetration behavior of Sylsens B through SC pre-treated (24 hours) with 1 % SLS at pH 5.2.
The accumulated amount of fluorescence (in AU) measured in the acceptor liquid after 24 hours at 657 nm upon excitation at 424 nm. Results as given mean ± standard deviations.
[a] Significantly different compared to sodium citric acid buffer $t(9) = 2.473$, $P = 0.0354$ and compared to untreated SC $t(9) = 2.486$, $P = 0.0347$.
n.p: no Sylsens B present.

DISCUSSION

The main goal of this study was to examine the skin penetration behaviour of a previously successful Sylsens B formulation (pH 5.2) that could be used in future clinical PDT experiments. Next to the healthy dermatomed skin, we used isolated SC because the SC forms the main skin barrier for penetration. (20-23)
We observed no penetration in healthy skin of 160 µM solution of Sylsens B neither at pH 7.4 nor 5.2 with 160 µM Sylsens B. The positive charge of Sylsens B, the hydrophilic character and the relatively high molecular weight might contribute to these findings. (24,25) However, when the SC was infected with *T. rubrum* we observed some differences in Sylsens B penetration between pH 5.2 and pH 7.4. The 5-day fungal growth resulted in Sylsens B penetration at pH 7.4, but not at pH 5.2.
We demonstrated in our previous work that at pH 5.2 a selective binding of this photosensitizer to the fungus occurred. Apparently, the presence of fungal particles in SC offers an increased binding area for Sylsens B at pH 5.2 and there is no Sylsens B available to penetrate the damaged skin. At pH 7.4, the binding capacity of Sylsens B to fungal elements is lower and this makes the penetration of Sylsens B molecules possible. (12) Although the amount of Sylsens B that penetrated the skin barrier at pH 7.4 was calculated to be 0.1% of the applied concentration, this amount could still potentially affect the underlying healthy epidermal and dermal cell layers.

From these experiments we conclude that Sylsens B in the water solution with low ion concentration and pH 5.2 does not penetrate the stratum corneum, even in the presence of *T. rubrum*). The risk of a systemic effect of Sylsens B is therefore minimized and from this point of view this formulation seems to be safe for a future application in a clinical setting.
However, when the SC was pretreated with SLS, Sylsens B could easily penetrate even at pH 5.2. In this case, the skin barrier was strongly reduced and the binding groups for Sylsens B were missing because of the absence of fungal elements. Therefore, the use of a detergent like SLS before PDT with Sylsens B should be avoided.

ACKNOWLEDGEMENTS

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