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Permeation studies of novel terbinafine formulations containing hydrophobins through human nails *in vitro*

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ABSTRACT

Existing treatments of onychomycosis are not satisfactory. Oral therapies have many side effects and topical formulations are not able to penetrate into the human nail plate and deliver therapeutical concentrations of active agent *in situ*. The purpose of the present study was to determine the amount of terbinafine, which permeates through the human nail plate, from liquid formulations containing enhancers, namely hydrophobins A–C in the concentration of 0.1% (w/v). The used reference solution contained 10% (w/v) of terbinafine in 60% (v/v) ethanol/water without enhancer. Permeability studies have been performed on cadaver nails using Franz diffusion cells modified to mount nail plates and filled with 60% (v/v) ethanol/water in the acceptor chamber. Terbinafine was quantitatively determined by HPLC. The amount of terbinafine remaining in the nail was extracted by 96% ethanol from pulverized nail material after permeation experiment and presented as percentage of the dry nail weight before the milling test. Permeability coefficient (PC) of terbinafine from reference solution was determined to be 1.52E−10 cm/s. Addition of hydrophobins improved PC in the range of 3E−10 to 2E−9 cm/s. Remaining terbinafine reservoir in the nail from reference solution was 0.83% ($n = 2$). An increase of remaining terbinafine reservoir in the nail was observed in two out of three tested formulations containing hydrophobins compared to the reference. In all cases, known minimum inhibitory concentration of terbinafine for dermatophytes (0.003 μg/ml) has been exceeded in the acceptor chamber of the diffusion cells. All tested proteins (hydrophobins) facilitated terbinafine permeation after 10 days of permeation experiment, however one of them achieved an outstanding enhancement factor of 13.05 compared to the reference. Therefore, hydrophobins can be included in the list of potential enhancers for treatment of onychomycosis.

1. Introduction

Nail and hoof are two different modifications of claws occurred in mammalian evolution and were produced by differential growth (Spearman, 1985), thus these two biological materials show some structural similarities. The structure and properties of the human nail plate are elucidated with the development of new technologies and analysis techniques. Evidence that the nail plate is produced by the underlying matrix is obtained by the researchers Zaias and Alvarez in 1968 (Zaias and Alvarez, 1968). Commonly used values of growth rate are 3 mm per month for fingernails and 1 mm per month for toenails, which means that the complete renewal of fingernails is around two times faster than the complete renewal of toenails (Murdan, 2008). Desmosomes as a type of junction between the membranes of the cells forming the nail plate and between adjacent corneocytes all along the tortuous intracellular boundaries are described in the works of Caputo et al. (1982) and McCarthy (2004). Cells on the dorsal side of the nail are thinner than those on the ventral side and intracellular spaces are frequently observed (Achten et al., 1991). Nail plate mainly consists of keratin filaments and Garson et al. determined their orientation (Garson et al., 2000). The energy to cut nails transversely (3 kJ/m^2) is only half that is needed to cut them longitudinally (6 kJ/m^2), which is similar with values found for horse hoof (Farren et al., 2004). Mean carbon content in the fingernails of healthy adults is 45% and increases with aging. Females have more sulfur and less nitrogen, but the sulfur content does not change with aging (Dittmar et al., 2008). Understanding the structure and properties of the nail plate is fundamental for the development of potent drugs, which can be used in the treatment of nail diseases. Further, the impact of toxins and drugs on nails can be better understood. Thus, although dead tissue, the human nail plate can provide useful information for retrospective analysis. For example poisons such as thallium and arsenic are stored in nails and a large number of trace elements can be detected in nails giving a picture about pollution or level of exposure to toxins at the working place. Nail clippings as

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easy available and non-invasive samples can be utilized for measuring long-term control of blood glucose levels in diabetic patients or detection of hepatitis B virus DNA in positive patients (Daniel et al., 2004). Cancer chemotherapeutic agents can induce changes in the nail. Paclitaxel, methotrexate, or taxanes, which includes paclitaxel induce mostly but not only changes where cell proliferation occurs in the nail matrix (Uyttendaele et al., 2004). The affinity of some substances to accumulate in nails and/or alter the nail structure, beside the retrospective analysis and information about condition of human organism in general, can serve as a starting point for drug development in the treatment of nail diseases, such as onychomycosis. Onychomycosis is a common nail disease and the number of patients rises among the elderly population. This fungal nail infection needs a prolonged treatment period and has a low cure rate. Drake et al. conclude in their study with 258 subjects with a median age of 51.5 years that the mean duration of the nail disease is 9.5 years (Drake et al., 1998). Potent and the latest widely accepted antifungal agent terbinafine with its lipophilic and keratinophilic nature fulfills the requirements for an effective drug. Terbinafine was developed from naftifine, which was discovered serendipitously in 1974 during the synthesis of compounds active in the central nervous system at Sandoz research institute Wander (Berney and Schuh, 1978; Stütz, 1988). The mode of action for this synthetic allylamine involves inhibition of enzyme squalene epoxidase in fungal ergosterol biosynthesis, which induces accumulation of intracellular squalene and cell's death (Ryder, 1992). It was suggested that orally administered terbinafine reaches the nail plate by diffusion from the nail matrix and the nail bed (De Doncker, 1999). The molecular mechanism by which terbinafine inhibits the enzyme reminds unclear because structural information of squalene epoxidases is currently unavailable (Fuglseth et al., 2009). Terbinafine can be applied systemically or topically. Oral tablets are usually prescribed for the treatment of onychomycosis, while topical formulations are indicated for skin infections. Due to adverse effects topical therapy in onychomycosis treatment would be preferable. Although oral terbinafine is since 19 years present on the world market (Newland and Abdel-Rahman, 2009), by our knowledge there is no efficient and approved topical terbinafine formulation for the treatment of onychomycosis on the market yet, mainly because of the restrictive barrier properties of the nail plate. Amorolfine and ciclopirox have been approved for onychomycosis treatment in Europe and ciclopirox has been approved by the FDA (Elkeeb et al., 2010). Usually, the treatment includes oral and topical application in combination according to a time schedule. Currently, investigations of drugs to be applied topically are divided in several directions: (I) iontophoretic delivery, which was demonstrated in the work of Nair et al. (2009), (II) addition of chemical enhancers as it was shown by Brown et al. (2009), (III) investigation of novel pharmaceutical formulations/carriers, such as transfersomes licensed by Celtic Pharma or lacquer formulations (Lehman et al., 2005; Jan et al., 2008), (IV) physical removal of certain parts of the nail, for example, by forming microconduits in the nail plate (Boker and Burks, 2007), and (V) discovery of new drugs, such as AN2690 by Hui et al. (2007).

The purpose of the present work was to investigate the permeability of the antifungal drug terbinafine hydrochloride through the human nail plate from liquid formulations containing various enhancers. Terbinafine (written terbinafine in the text indicates used terbinafine hydrochloride) has been chosen because it is the most potent antifungal drug against dermatophytes (Ghannoum et al., 2000). Due to its lipophilicity, formulations were prepared with the addition of ethanol and therefore studies to evaluate a potential influence of ethanol on the human nail plate were performed. The amphiphilic fungal proteins called hydrophobins showed an enhancement effect in our previous permeability studies using caffeine as model drug (Vejnovic et al., 2010) and therefore it is hypothesized and tested if hydrophobins can be potential and promising universal enhancers to be applied to the human nail plate. Thus, three different hydrophobins, small proteins with astonishing features of self-assembling properties were used in this study and permeability coefficients of terbinafine were determined with their presence using human cadaver nails. The relation between the amounts of terbinafine penetrated into the nail, which forms a reservoir in the nail and enhancement factor was investigated after the permeation experiment and performed milling test. Finally the formulations were discussed in respect to their enhancement factors and known minimum inhibitory concentration of terbinafine for dermatophytes.

2. Materials and methods

2.1. Materials

Terbinafine hydrochloride was purchased from Molekula, Germany and hydrophobins A–C were a gift from CIBA, Switzerland. Hydrophobin A is a class I hydrophobin TT1 from the thermophile fungus *Talaromyces thermophilus* produced in *E. coli* as a fusion protein with glutathione-S-transferase. Hydrophobin B is a chimeric protein consisting of the N-terminal part of the class I hydrophobin SC3 from *Schizophyllum commune* and the C-terminal portion of the class II hydrophobin HFB2 from the fungus *Trichoderma reesei*, also expressed in *E. coli*. Hydrophobin C is a class I hydrophobin POH3 from the fungus *Pleurotus ostreatus* produced in *E. coli* as a fusion protein with glutathione-S-transferase. 96% (v/v) ethanol (Ph.Eur. III) was purchased from Synopharm, Basel, Switzerland. Double distilled and filtrated water was produced in-house. All other reagents were of analytical grade.

2.2. Collection and characterization of nail samples

Human cadaver nail samples were collected from corpuses at the Institute of Anatomy and Cell Biology, Freiburg, Germany. Method of collection was developed in a previous study and is explained in detail elsewhere (Vejnovic et al., 2010). Information about age and sex were recorded for all nail samples and then kept at −20 °C. One day before the permeability studies were performed, nail samples were left over night for equilibration at open air and room temperature. After equilibration nail samples were characterized by the measurement of weight (Analytical balance, type AT261, Mettler Toledo, Switzerland), thickness (Digit cal SI, TESA S.A., Switzerland), transonychial water loss (Tewameter TM210, Courage&Khazaka electronic GmbH, Germany), and visioscan images (Visioscan VC98, Courage&Khazaka electronic GmbH, Germany). Subsequently, they were hydrated in double distilled and filtrated water for 60 min in order to achieve maximal hydration. Thickness and visioscan images of swollen nails were recorded. The weight of cut nail used in the experiment was calculated from difference in weight of the whole nail sample and the nail rest after cutting. Transepidermal water loss indicates the integrity and state of the skin and can be measured with the Tewameter. The same method applied to the nail delivers useful information about nail state and hydration rate. Transonychial water loss (TOWL) of dry nails and wet nails mounted in Franz diffusion cells without and with 60% (v/v) ethanol/water in the acceptor chamber was measured. The distance of 7.78 mm between the sample and the sensors in the measuring sonde, which represents the thickness of a gum ring of the modified Franz diffusion cell set up, was kept constant. Visioscan VC98 is equipped with an UV-A light video camera and it was originally developed to study skin surface characteristics. All characterization measurements were repeated directly after the permeation experiments and once again after 24 h.