Abstract
This study investigated the *in vitro* nail permeability of penetrants of varying lipophilicity—caffeine (CF, log *P* –0.07), methylparaben (MP, log *P* 1.96) and terbinafine (TBF, log *P* 3.3) and the effect of 2 novel penetration enhancers (PEs), thioglycolic acid (TA) and urea hydrogen peroxide (urea H2O2) on their permeation. Studies were conducted using full thickness human nail clippings and ChubTur® diffusion cells and penetrants were applied as saturated solutions. The rank order of steady-state penetrant flux through nails without PE application(MP > CF > TBF) suggesteda greater sensitivity to penetrant molecular weight rather than log *P*. TA increased the flux of CF and MP ~4- and ~2-fold, respectively, whilst urea H2O2 proved ineffective at enhancing permeability. The sequential application of TA followed by urea H2O2 increased TBF and CF flux (~19- and ~4-fold, respectively) but reversing the application order of the PEs was only mildly effective at increasing just MP flux (~2-fold). Both nail PEs are likely to function via disruption of keratin disulphide bonds and the associated formation of pores that provide more 'open' drug transport channels. Effects of the PEs were penetrant specific, but the use of a reducing agent (TA) followed by an oxidising agent (urea H2O2) dramatically improved human nail penetration.

1. Introduction

The human nail plate is the most prominent part of the nail structure. It functions to protect the tips of the fingers and enables tasks such as the manipulation of small objects and scratching. The packing of dead, cornified cells in the nail plate gives rise to distinct dorsal, intermediate and ventral layers. The thickness of these layers varies between individuals, but the whole nail is generally between 0.5 and 1.0mm thick. It is composed of _-keratin, which provides structural rigidity, lipid and water (Gupchup and Zatz, 1999).

The human nail is an excellent barrier against the ingress of foreign material, but as a consequence it also prevents effective topical treatment of ungual disorders such as onychomycosis (Einarson et al., 1996; Elewski, 1998; Seebacher, 2003). When infection resides in the nail plate, nail bed, or both, therapeutic antimicrobial drug concentrations must be achieved in the nail bed for treatment to be effective (Elewski, 1998). However, this is difficult to achieve because the permeation of agents into the nail is low. Permeation occurs via passive diffusion with the rate determined by the physicochemical properties of the compound. Unfortunately, the most efficacious treatments for nail disorders do not penetrate the nail plate in sufficient amounts to be clinically effective (vanHoogdalem et al., 1997; Traynor et al., 2008).

The poor clinical response to topical formulations has resulted in oral dosing remaining first-line therapy for many ungual disorders. However, oral treatment regimens can take months to be effective because they require large amounts of the drug to be loaded into the systemic circulation in order to achieve a local therapeutic concentration in the nail bed. Furthermore, systemic therapy may be contraindicated and has the potential for side-effects. For example, the oral antifungal agent terbinafine, which is used for the treatment of onychomycosis can cause serious hepatotoxic side-effects and patients require continuous monitoring for the duration of the therapy (Chambers et al., 2001; Ajit et al., 2003). The inadequacies of the current treatments mean that there is still a requirement to develop an effective topical treatment for ungual disorders that will allow

site-specific administration and minimise the systemic exposure of the therapeutic agent.

To date, strategies employed to increase the permeation of therapeutic agents across the nail plate can be categorised into mechanical or chemical methods. Mechanical methods can be effective, but often involve damage or partial removal of the nail plate. Kobayashi et al. (1999) showed that removal of the dorsal layer of the nail plate before drug application increased the ungula permeation of a compound irrespective of itswater solubility.However, patient compliance tends to be low when such aggressive methods are used as part of topical antifungal therapy (Grover et al., 2007).

Chemical methods of nail penetration enhancement involve the use of penetration enhancers (PEs) and numerous studies have demonstrated the effectiveness of PEs *in vitro*. Sulfhydryl (SHcontaining) compounds have been used to increase itraconazole uptake by the nail and acetylcysteine has been used to increase the ungual retention of miconazole nitrate (Sun et al., 1999). Dimethylsulfoxide enhances the delivery of ketaconazole and salicylic acid (Hui et al., 2002), and 2-mercaptopyridine-1-oxide, N-(2-mercaptopropionyl) glycine (Malhotra and Zatz, 2002) and 2- *n*-nonyl-1,3-dioxolane (SEPA®) (Hui et al., 2003) are also reported to be effective PEs. Few, if any, of these agents, however, have been used in commercial products and very little information is available on the mechanism by which they function. One exception is thioglycolic acid (TA) which has been shown to disrupt the disulphide bond network of hair keratin (Olsen, 1999). Recent work has postulated that ungual keratin disruption caused by the application of TA leads to elevated levels of nail swelling. In addition, caffeine permeability experiments have demonstrated that pretreating nail samples with TA increases ungual drug flux (Khengar et al., 2007).

One reason why ungual PEs have not been comprehensively and systematically investigated previously is the lack of appropriate *in vitro* assessment methods. Many of the current methods employed to investigate permeation through the nail are time consuming or difficult to perform and the generation of significant data can take weeks or months. However, a novel nail diffusion model (the ChubTur® cell) has been developed by MedPharm Ltd. (Guildford, UK) that allows the assessment of nail penetration using 3mm×3mm distal nail clippings (Khengar et al., 2007). The ChubTur® model, specifically designed for monitoring the *in vitro* ungual permeation and deposition of topically applied drugs, solves in part, the problems associated with existing *in vitro* models as it requires a relatively modest sample size and thus facilitates the use of human nail material over animal derived substitutes even though the latter are usually more readily available. The ChubTur® system therefore allows the investigation of ungual delivery under an environment that closely mimics the *in vivo* situation.

In the present study the ChubTur® cell was utilised to determine the influence of log *P* on flux through healthy human nails and to investigate the effectiveness of TA and urea hydrogen peroxide (urea H2O2) at enhancing ungual flux. An additional objective was to confirm whether enhanced ungual flux could be further improved by sequentially pre-treating nail samples with both PEs and whether the PE pre-treatment order influenced any additive or synergistic effects by altering the order of PE application. The final objective of the present study was to seek to identify if any of the PE effects were drug specific. Three permeants were selected for the present study. Two were model

penetrants caffeine (CF) (log *P* –0.07 MWt 194) and methylparaben (MP) (log *P* 1.96 MWt 152), and the third was a relevant therapeutic agent, terbinafine hydrochloride (TBF) (log *P* 3.3 MWt 328) an antifungal allylamine which is used in the treatment of onychomycosis. Although the permeating compounds were not of identical molecular weight (due in part to their contrasting lipophilicities), it was anticipated that the influence of this variantwould be relatively minor compared to that of the permeant partition coefficient. The two PEs used in this study were selected on the basis of previous investigations which demonstrated their potential application as ungual PEs (Khengar et al., 2007). The first PE was TA, a sulfhydryl-containing compound, and a well characterised reducing agent (Anfinsen and Haber, 1961; Kuzuhara and Hori, 2003). The second PE was urea H2O2, a strong oxidising agent shown to increase nail swellability in a similar manner to TA (Khengar et al., 2007), and is currently utilised commercially by the hair care industry and also forms part of some tooth bleaching products (Wickett, 1987; Li, 1996). It is anticipated that insights into nail permeation enhancement will allowthe subsequent rational design of a clinically effective topical formulation for the treatment of ungual disorders such as onychomycosis.

2. Materials and methods

2.1. Materials

Human nail clippings were donated from healthy volunteers following approval by the King's College Research Ethics Committee (Study ref no. 04/05-126). Calibrated ChubTur® permeation cells were kindly donated by MedPharm Ltd. TA, urea H2O2, caffeine and methylparaben were purchased from Sigma–Aldrich (Dorset, UK). Phosphate buffered saline (PBS) (0.15 M, pH 7.3) was from Oxoid (Hampshire, UK). Ethanol (EtOH) was purchased from BDH Chemicals Ltd. (Dorset, UK). Scintillation fluid (hionic fluor) was supplied by PerkinElmer (Bucks, UK). Tritiated water (3H2O) was purchased from Amersham Biosciences (Buckinghamshire, UK). Triethylamine, orthophosphoric acid, potassium dihydrogen orthophosphate (KH2PO4) and acetonitrile (ACN) were supplied by Fisher (Leicestershire, UK). Terbinafine hydrochloridewas supplied by QueMaCo Ltd. (Nottingham, UK).

2.2. Permeation studies

Human nail clippings obtained from 15 donors, aged between 18 and 65 years, were washed with 70% EtOH (v/v) then deionised water 3 times. They were allowed to dry overnight in an open Petri dish at room temperature after which theywere either used immediately or stored in a clean glass container at 4 ◦C. Nail clippings were mounted on to pre-calibrated ChubTur® cells, as described previously (Khengar et al., 2007). Nail sections (approximate size 3mm×3mm) were clamped and held in place between receiver and donor chambers. The dorsal nail surface area exposed to the donor chamber was 0.05cm^2.

The concentrations of the PEs used in this study were based on earlier work (Khengar et al., 2007). TA (5%, w/w) was prepared in 80:20 water/EtOH (v/v) solution. Urea H2O2 solution was prepared in water (Millipore) such that the concentration of the H2O2 was 17.5% or 15% (w/v) as required. If no PE was applied, 80:20 water/EtOH (v/v) was used to hydrate the nails over 20 h (there was no significant increase in nail hydration beyond this time, data not shown). When applying a single PE, either 5% TA (w/w) or urea

H2O2 solution was applied (0.5 ml) to the donor compartment of the ChubTur® cell so that the dorsal surface of the nail was submerged. Following a period of 20 h, the PE solution was removed and the donor compartment was flooded with deionised water to wash away any residual PE. The deionisedwaterwas then removed afterwhich thewashing stepwas repeated a further two times with fresh deionised water. Any remaining moisture was removed using a dry tissue towel. If nails were to be treated with more than one PE, the second PE (either 5% TA (w/w) or urea H2O2 solution) was applied in the sameway again for 20 h, following the removal of the first PE and washing procedure. The second PE was again washed out of the donor chamber and any excess solution was removed using tissue towels. The receiver compartments of each cell were then filled to the calibration mark with the relevant receiver fluid; for CF thiswas PBS, forMP thiswas 80:20 PBS/EtOH (v/v) and for TBF the receiver fluid was 50:50 PBS/EtOH (v/v) to maintain sink conditions throughout the experiment (PBS was 0.15M and pH 7.3). The stability of the compounds in the relevant receiver fluid was confirmed over a period of 30 days (data not shown). Penetrant solutions were applied (0.5 ml) to the dry clean donor compartment after the application of the PEs or vehicle control. Penetrants were prepared and used at a saturated concentration to maintain a constant thermodynamic activity. Saturated solutions of CF, MP and TBF in PBS, 80:20 PBS/EtOH and 50:50 PBS/EtOH, respectively, were prepared by adding an excess of solid compound to solvents (20 ml) and stirring until saturation was achieved (determined by HPLC) followed by filtering using a 0.45_msyringe filter (Nalgene, Hereford, UK) at room temperature. The diffusion cells were sealed with parafilm for the duration of the experiment to prevent evaporation and incubated in a water bath maintained at 32 ◦C in order to keep the enclosed dorsal nail surface temperature at approximately 28 ◦C (verified using a k-type insulated wire probe (Hanna Instruments, Bedfordshire, UK)) the temperature of the extremities such as fingers and toes in human beings under normal conditions (Maddock and Coller, 1933).

2.3. Diffusion cell integrity verification

Following each permeation study, an integrity check was performed using 3H2O as a well characterised permeability marker. The donor compartments of cells were washed by flooding with deionised water 3 times and then dried using clean tissue towels. 3H2O (3.7 Bq/ml) was applied to the donor chamber of each cell after replacing the fluid in the receiver chamber with deionised water. Samples (1 ml) were removed from the receiver chambers of cells after 1 h and 20 h and were aliquoted directly into scintillation vials to which 4 ml of scintillation fluid was added. The vials were closed, mixed thoroughly by shaking and analysed using a LS 6500 Multipurpose scintillation counter (Beckman CoulterTM, Buckinghamshire, UK). Radioactivity was quantified as disintegrations per minute (DPM) which are independent of quenching effects observed in liquid scintillation (Cammen, 1977). Cellswere rejected if total DPM at 1 h were on average more than 20% higher than the mean of the other cells that had undergone the same pre-treatment or if the total DPM at 20 h was on average more than 40% higher than the mean of the other cells that had undergone the same pretreatment. These valueswere used aswewould not expect nail drug diffusion variability to exceed 20% after 1 h or 40% after 20 h when a varied nail sample pool is utilised (Khengar et al., 2007).

2.4. Data analysis

Cumulative amounts of compound (_g) penetrating the nail per surface area (cm2)were corrected for previous sample removal and plotted against time (h). The slope of the linear portion of the permeation rate profile ($R2 \geq 0.95$) was estimated as the steady-state flux (Js) of penetrant permeation. Lag time was derived from the x intercept of the slope at steady state. Enhancement ratio (ER) was calculated using equation (1):

$$ER = Js(E)/ Js(C) \quad (1)$$

where $Js(E)$ and $Js(C)$ are the flux values of the penetrant following pre-treatment with enhancer or control (no pre-treatment),respectively. Results were expressed as the mean±standard deviation (SD) of 3–8 determinations.

A one-way ANOVA was used for statistical analysis of variance and differences were further evaluated for significance with a post hoc Tukeymeans comparisons test. A level of $P \leq 0.05$ was considered significant.

2.5. HPLC analysis

A series 200 LC pump with autosampler and vacuum degasser connected to a UV/Vis detector 785A (PerkinElmer, Bucks, UK) was used for analytical determination of CF and MP. This system was connected, via a Nelson network chromatography interface (NCI) 900 and Nelson 600 Series LINK to a PC with Turbochrome Navigation software (PerkinElmer, Bucks, UK) for data collection and interpretation. For the analysis of TBF, a Waters 2487 dual _ absorbance detector, 600 controller, 717 plus autosampler, and Millennium32 Chromatograph Manager Software were used. HPLC analysis parameters are detailed in Table 1. Analysis of CF and MP was adapted from the method described by Akomeah et al. (2004) and the method for TBF was based on that described by Denouel et al. (1995). The HPLC methods were deemed to be fit for purpose upon assessment of linearity, precision, accuracy and asymmetry according to the current ICH guidelines (ICH Q2A 1995; ICH Q2B 1997).

3. Results

3.1. Permeability through untreated nails

The *in vitro* flux (at steady-state) of the 3 penetrants through nails that were not treated with the PEs were significantly different ($P \leq 0.05$, ANOVA) and ranked MP> CF > TBF (Figs. 1–3). The rate of MP flux was approximately 3-fold and 11-fold greater than that of CF and TBF, respectively. The rate of CF ungual flux was around 7 times greater compared to the flux of TBF. The mean lag times calculated for each penetrant, *i.e.* the time taken to reach steady-state permeation,were inversely related to their permeability (MP≤CF < TBF) but the difference between MP and CF was not significant ($P > 0.05$).

3.2. Permeability following single PE application

The flux of the 3 penetrants was influenced to different extents as a result of nail pre-treatment with the PEs (as reflected by the ER values, Table 2). Pre-treating nails with TA alone significantly enhanced ($P < 0.05$) the ungual flux of CF and MP (Figs. 1 and 2) whilst the increase in TBF permeation (Fig. 3) was not significant ($P > 0.05$). When urea H2O2 was applied as the sole PE, the flux of all 3 penetrants were statistically equivalent ($P > 0.05$) to their respective flux through untreated nails. The lag time for all

penetrants was effectively reduced as a result of TA pre-treatment, whereas no significant decreases ($P > 0.05$) were observed when
urea H2O2 was the PE.

Table 1 HPLC parameters for analysis of caffeine (CF), methylparaben (MP) and terbinafine hydrochloride (TBF).

Parameter	CF	MP	TBF
Column specification	Phenomenex® LunaTM 5_mC18 column (150×4.6mm)	Phenomenex® HypersilTM 5_mC18 column (150×4.6mm)	Phenomenex® LunaTM 5_mC18 column, 150mm×2.0mm) Guard column: C18 4.0mm×2.0mm
Injection volume	50 µl	20 µl	40 µl
Mobile phase composition	92:8 80:20 60:40 92% (0.05 M KH2PO4; 1% triethylamine; pH 3.5) 8% acetonitrile	80% (0.05 M KH2PO4; 1% triethylamine; pH 3.5), 20% acetonitrile	60% (Triethylamine and orthophosphoric acid buffer; pH 2), 40% acetonitrile
Flow rate	1 ml/min	1ml/min	0.3 ml/min
Run time	20 min	15 min	10 min
Detection	λ=275nm	λ=254 nm	λ=224nm

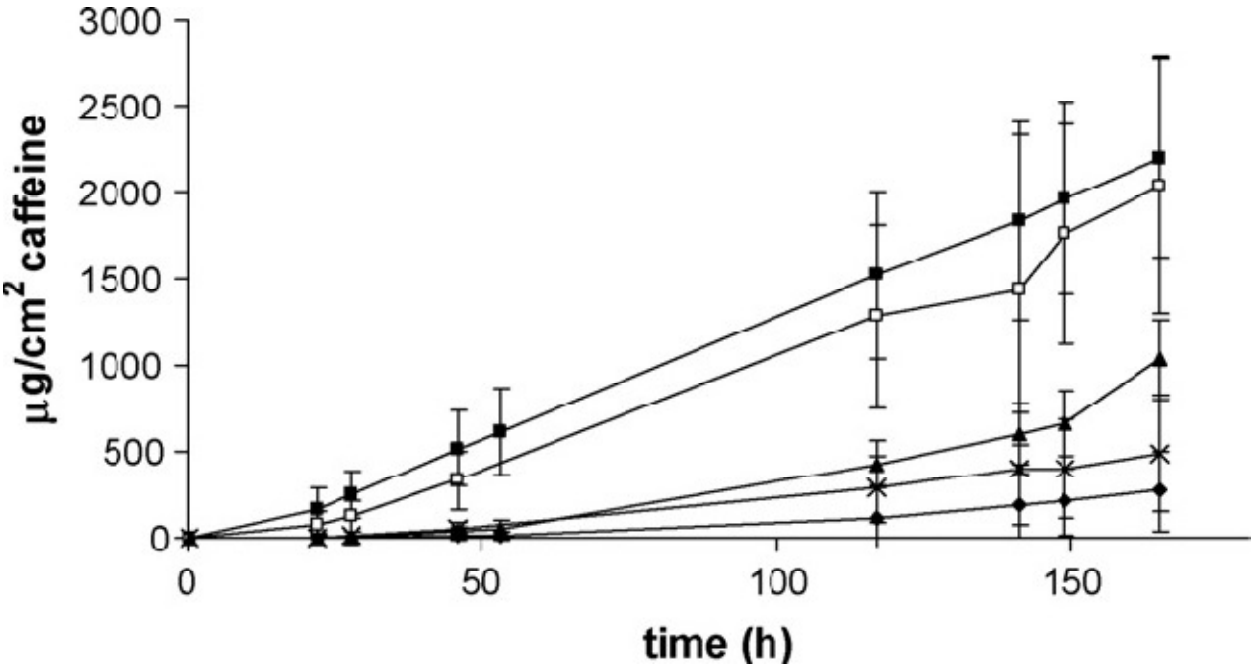

Fig. 1. Permeation profiles of caffeine following pre-treatment with single and sequential penetration enhancing systems. Single treatment consisted of 20 h pretreatment with either thioglycolic acid (TA) (_) or urea hydrogen peroxide (urea H2O2) (_). Sequential treatment systems were 20 h TA pre-treatment followed by 20h urea H2O2 (_) and 20 h urea H2O2 pre-treatment followed by 20 h TA pretreatment (×). Control (_) represents no penetration enhancer (n = 3–8, mean±SD).

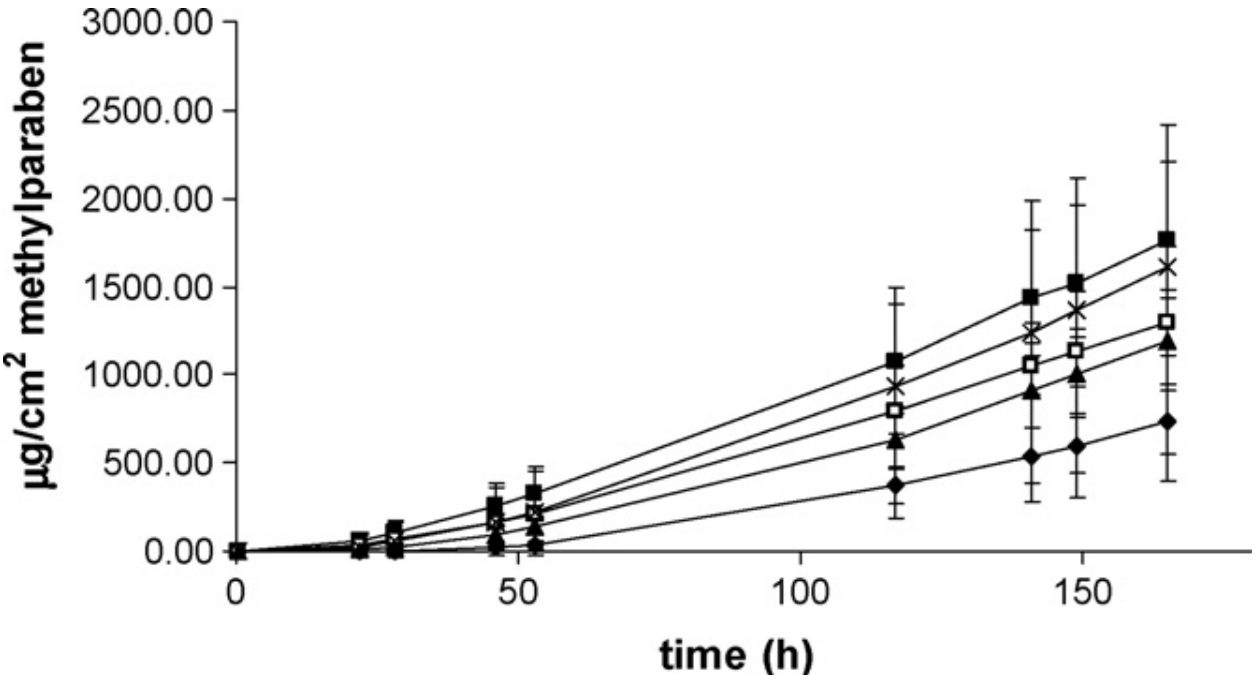

Fig. 2. Permeation profiles of methylparaben following pre-treatment with single and sequential penetration enhancing systems. Single treatment consisted of 20 h pre-treatment with either thioglycolic acid (TA) (_) or urea hydrogen peroxide (urea H2O2)

(_). Sequential treatment systemswere either 20 h TApre-treatment followed by 20 h urea H2O2 (_) or 20 h urea H2O2 pre-treatment followed by 20 h TA pretreatment (×). Control (_) represents no penetration enhancer (*n* = 6–7,mean±SD).

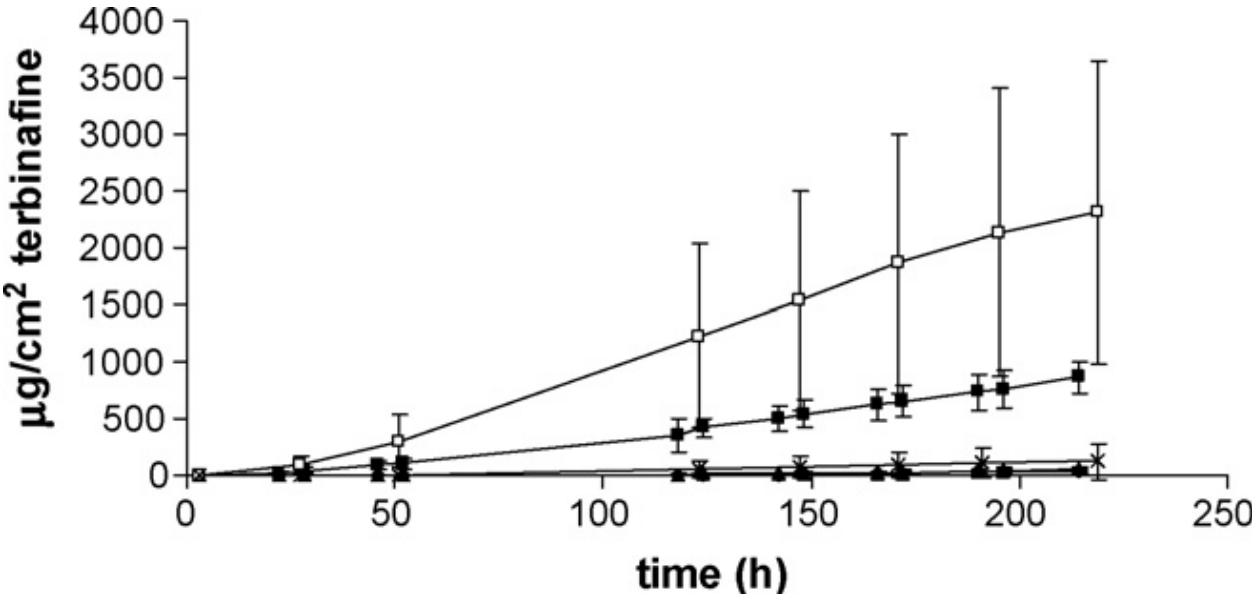

Fig. 3. Permeation profiles of terbinafine hydrochloride following pre-treatment with single and sequential enhancing systems. Single treatment consisted of 20 h pre-treatment with either thioglycolic acid (TA) (_) or urea hydrogen peroxide (urea H2O2) (_). Sequential treatment systems were either 20 h TA pre-treatment followed by 20 h urea H2O2 (_) or urea H2O2 20 h pre-treatment followed by 20 h TA pre-treatment (×) (the data for this is not easily visible on the graph as the results overlap with control data). Control (_) represents no penetration enhancer (*n* = 6–8, mean±SD).

Table 2 The flux rates (µgcm−2 h−1, mean±SD) of caffeine (CF), methylparaben (MP) andterbinafine hydrochloride (TBF) through nails pre-treated with thioglycolic acid (TA) and urea hydrogen peroxide (urea H2O2) as part of the single or sequential pretreatment regime are given together with lag times (h, mean±SD), (*n* = 3–8).

Drug	Penetration enhancer	Flux (µgcm−2 h−1)	Lag time (h)	ER
CF	None	3.64 ± 1.54	70.70 ± 34.29	–
	5% TA	14.10 ± 3.42*	10.71 ± 10.63	3.87
	15% Urea H2O2	8.45 ± 3.76	52.64 ± 20.25	-
	5% TA followed by 15% Urea H2O2	14.16 ± 4.98*	23.57 ± 10.59	3.89
	15% Urea H2O2 followed 5% TA	3.76 ± 2.24	45.45 ± 16.87	–
MP	None	6.05 ± 1.48	50.38 ± 3.36	–
	5% TA	12.51 ± 4.29*	29.65 ± 3.55	2.07

	15% Urea H2O2	9.27 ± 1.93	42.31 ± 5.47	–
	5% TA followed by 15% Urea H2O2	9.72 ± 6.36	37.25 ± 13.10	–
	15% Urea H2O2 followed 5% TA	12.17 ± 2.98*	37.74 ± 3.76	2.01
TBF	None	0.55 ± 0.71	121.39 ± 14.13	–
	5% TA	4.73 ± 1.01	33.62 ± 14.74	–
	17.5% Urea H2O2	0.43 ± 0.29	113.33 ± 10.64	–
	5% TA followed by 17.5% Urea H2O2	10.22 ± 5.22*	26.91 ± 27.16	18.67
	17.5% Urea H2O2 followed by 5% TA	1.25 ± 0.80	53.93 ± 22.81	–

Flux derived from linear plot ($R2 \geq 0.95$).
Enhancement ratios (ER) are only given where penetrant flux increase was significant ($P \leq 0.05$) compared to untreated nails.
* Increase is significant when compared to the control value.

3.3. Application of both PEs in sequence and the effect of application order

The flux of 2 of the 3 penetrants (CF and TBF) was significantly higher ($P < 0.05$) through nails pre-treated with TA followed by urea H2O2 compared to untreated nails. The increase in TBF permeation resulted in the highest observed penetrant flux increase (ER = 18.67). The time taken for steady-state flux to be achieved however was significantly decreased for all penetrants as a result of this sequential pre-treatment regime. In addition, the rate of TBF fluxwas significantly greater ($P < 0.05$) across nails pre-treated with TA followed by urea H2O2 compared to TA-only pre-treated nails (approximately double).

Reversing the application order of the PEs (pre-treating with urea H2O2 first and TA second) showed considerable differences in the flux enhancement of all 3 penetrants. The flux of MP alone was significantly higher ($P < 0.05$) across nails pre-treated with urea H2O2 followed by TA compared to untreated nails, but the increased rate of MP permeation was not significantly greater ($P > 0.05$) than the enhanced MP flux through TA-only pre-treated nails. Significant reductions in lag times were however observed for MP and TBF when nails were pre-treated with urea H2O2 followed by TA (a reduction of 25% and 78%, respectively).

4. Discussion

The human nail plate is composed of three compacted keratinized epithelial cell layers (the dorsal, intermediate and ventral) (Lewis, 1954;Dawber, 1980; Gupchup et al., 1999). As an adsorption barrier the nail has been simplistically described in the literature as a hydrophilic gelmembrane (Mertin and Lippold, 1997b; Kobayashi et al., 1999) that effectively obstructs drug permeation by virtue of its chemical composition (Gniadecka et al., 1998). Extensive folding of the ‘hard’ keratin protein chains of the nail aremaintained by extremely stable disulphide bonds whichcan bemodifiedusing oxidizing or reducing conditions. Disruption of these disulphide bonds is hypothesised to compromise the structure of proteins that have a high proportion of disulphide cross-links. This theory has already been exploited by the cosmetics industry to produce hair straightening and waving agents (disulphide bond reduction and oxidation) (Wortmann, 1994; Robbins, 2002) and depilatory products (disulphide reduction) (Liew, 1999).

Permeation through the nail is generally believed to be influenced by the physicochemical properties of the penetrating molecule. Nail permeability has been previously been reported to be especially sensitive to the lipophilicity of a drug (Walters et al., 1983;Kobayashi et al., 1999). In the present study, the permeation of three compounds that differed considerably in terms of lipophilicity was investigated—CF, (log *P* –0.07, MWt 194), MP (log *P* 1.96, MWt 152), and TBF (log *P* 3.3, MWt 328). The rank order of *in vitro* penetrant permeation through nails that were not pre-treated with PEs however, displayed an inverse correlation to molecular weight rather than log *P*. This finding may indicate that the molecular weight of acompound traversing the nail plate has greater influence than its lipophilicity in determining the extent of its permeation. The present study therefore, supports previous claims that molecular weight is the critical physicochemical property in ungual drug permeation (Mertin and Lippold, 1997a; Kobayashi et al., 2004) and proposes that the gel membrane description commonly given to the nail is overly simplistic and that nail barrier properties might be better likened to that of a more complex compact, hydrophilic filter.

An alternative explanation for the permeation rank order of the 3 penetrants through untreated nails is that drug retention or binding in the nail keratin matrix occurred after drug penetration. The retention of various drugs by skin is well documented (Hashiguchi et al., 1998; Akomeah et al., 2004), for example polar drugs such as doxycycline are reported to bind to skin keratin via hydrogen bonds (Banning and Heard, 2002). Similarly, for the nail, there are reports that antifungal agents including TBF have a strong binding affinity for keratin (Tatsumi et al., 2002). It is possible therefore, that the ungual flux of CF,MP and TBF through untreated nails demonstrate the extent of drug binding in addition to the influence of penetrant MW and log *P*.

The nature of the application vehicle in which the penetrating drug is dissolved has also previously been reported to effect ungual permeability. Aqueous vehicles have been shown to facilitate drug transport across the nail by enhancing nail swelling which presumably results in greater drug mobility in the barrier and perhaps the formation of pores (Murdan, 2002). The hydration state of the nail therefore, is undoubtedly an important factor in compound flux, and has been demonstrated by numerous researchers (Wessel et al., 1999; Gunt and Kasting, 2007). Due to their contrasting solubilities, each of the applied penetrants used in the present study were solubilised in vehicles that differed in their aqueous and ethanolic content (CF–PBS, MP–80:20 PBS:EtOH, v/v and TBF–50:50 PBS:EtOH, v/v). In addition, to ensure sink conditions were maintained during the permeation experiments the receiver fluids for each permeation study was matched to respective application solvent. Ethanol enhances the skin permeation of topically applied agents (Megrab et al., 1995; Stinecipher and Shah, 1997) by modifying the lipid domains of the stratum corneum (Bommannan et al., 1991;Williams, 2003). As the lipid content of the nail is considerably less than skin (the dorsal nail layer contains only ~0.1% lipid compared to ~20% in skin) the necessity of having to use different PBS/ethanol mixtures in the permeation studies of the current investigation was anticipated to have little or no effect on ungula penetrant mass transfer. This postulation is supported by previous studies which confirm that the swelling behaviour, and hence the hydration state of the nail is not significantly different ($P > 0.05$, ANOVA) in mixed ethanol/aqueous solvents consisting of 0%, 20% and 50% ethanol (Khengar et al., 2007). Therefore, whilst aqueous vehicles clearly assist ungual

permeation, the penetrating enhancing ability of ethanol is likely to be absent in the nail at the levels used in this study, despite its ability to act as a dermal enhancer.

The use of chemical nail penetration enhancers to deliver therapeutically relevant concentrations of drugs to the nail unit has recently received considerable interest (vanHoogdalem et al., 1997; Hui et al., 2003; Mohorcic et al., 2007; Khengar et al., 2007; Hao et al., 2008), largely due to the ineffective penetration of topically applied antimycotics through the nail plate. The present investigation considered the potential of TA and urea H2O2 to enhance the ungual permeation of CF,MP and TBF as these two putative PEs have previously been shown to elevate nail hydration following a simple incubation protocol of the nail with the PE (Khengar et al., 2007). It was hypothesised that both PEs encourage nail swelling which leads to a structural expansion of the nail membrane. The chemical bond disruption facilitates the uptake of unexpectedly high quantities of water after PE application which was proposed to enhance drug mobility in the barrier. In the present study, TA successfully enhanced the ungual flux of CF and MP and reduced the lag times for all 3 penetrantswhilst urea H2O2 was less effective. TA and salts of TA are commonly used in depilatory products at a concentration of 5% (Olsen, 1999) and in hair waving or straightening products at concentrations between 8 and 11%. Likewise, urea H2O2 is a component of some tooth bleaching formulations and concentrations used commercially vary between 10 and 35% (Li, 1996; Oltu and Gurgan, 2000). TA contains a thiol moiety that enables the chemical break down of disulphide linkages in hair and wool through a reduction process (Gupchup et al., 1999). Due to the similarities in the amino acid profiles of hair, wool and nail (Robbins, 2002) it is logical to hypothesise that a similar redox reaction involving nail disulphide bond reduction by TA compromised the rigidity of the ungual barrier, permitting an improvement in the rate of CF and MP flux. In contrast to TA, urea H2O2 is an oxidising agent, and as a hydrogen-bonded adduct it is reported to be more stable thanH2O2 alone (Gonsalves et al., 1991). Although disulphide oxidation is possible in the presence of excess oxidant (Capozzi and Modena, 1974), there have been, to our knowledge, no reports of oxidising agents influencing the barrier properties of the human nail. In this study, pre-treating nails with urea H2O2 did not increase the permeation of CF, MP or TBF, perhaps due to the marked stability of the disulphide bond against oxidation in comparison to its reactivity with the thiol group (Capozzi and Modena, 1974; Gilbert, 1995).

The reduction of disulphide bonds is a reversible process and broken disulphide linkages can be reformed using an oxidizing agent (Wickett, 1987); a fact that suggests that applying an oxidizing agent to the nail directly after a reducing agent would be ineffective at enhancing ungual permeability and might instead restore barrier function. However, in the present study permeation of 2 of the 3 penetrants was increased as a result of applying the PEs in sequence; CF and TBF permeation clearly improved when nails were pre-treated with TA followed by urea H2O2 (~4- and 19-fold, respectively). This could be explained by the relatively high concentration of urea H2O2 applied to the nail as typically, only 2–3% solutions of H2O2 are used to reinstate reduced disulphide links in human and sheep hair (Marshall et al., 1991; Robbins, 2002), whilst a much higher concentration of 15 or 17.5% was applied to nails in the current investigation. In addition, the reformation of broken disulphide bonds in human hair is reported to be only

85–95% efficient (Cannell, 1988), and is believed to be accompanied by the formation of mixed disulphide bonds or cysteic acid. An excess of urea H2O2 remaining in the nail following its application may promote the oxidation of mixed disulphides or even, as previously reported, lead to an oxidation cascade starting with disulphide bond reformation followed by the sequential production of thiosulfinates, thiosulfonates and ultimately sulfonic acids (Capozzi and Modena, 1974).

The selection of TBF as a model penetrant in the present study was anticipated to illustrate the permeability potential of this lipophilic drug through the nail compared to drugs of contrasting log *P* and in addition, to directly assess whether the delivery of this relevant therapeutic antifungal compound through the nail could be enhanced. Excellent retention of TBF has been reported in human and animal fat tissues in clinical pharmacokinetic studies, reflecting the high lipophilicity of this drug (Jensen, 1989). Based on the hydrophilic-like nature of the nail membrane (Walters et al., 1983;Walters, 1985; Kobayashi et al., 1999) and the low lipid content of the nail, TBFwould be expected to permeate the nail poorly. In the present study however, the steady-state flux of TBF was enhanced to the greatest extent following nail pre-treatment with TA followed by urea H2O2, the rate being comparable to the highest observed flux of the study (flux of hydrophilic CF after the same pre-treatment, Table 2). This surprising result might be explained by the expanded, ‘swollen’ nail keratin network induced by PE pretreatment which may have facilitated TBF permeation. Although pre-treated swollen nails would be likely to have a hydrophilic internal environment, it is proposed that using a mixed aqueous/ ethanolic solvent for TBF studies (50:50) facilitated partitioning of this drug whilst still having a high enough content of aqueous matter to maintain the increased open pore state of the nail. Whilst this is a logical hypothesis, this data clearly raises important questions about nail permeability and nail biochemistry in general and these issues warrant further investigation.

MP was the only penetrant to see increased permeation when the PEs were applied in the reverse sequence (urea H2O2 followed by TA), and although it is at present unclear why this was the case, this finding demonstrates that the effectiveness of the sequential pre-treatment regime is compound-specific and the need to confirm the efficacy of the pre-treatment with respect to a relevant therapeutic molecule prior to its incorporation into a topical ungula formulation.

5. Conclusions

Nail PE systems consisting of TA and urea H2O2 were tested for their effectiveness at enhancing the permeation of 3 model penetrants with a range of lipophilicity. The permeation enhancement of each penetrant following PE treatment was compound specific and the most effective enhancement was for TBF permeation which resulted from sequential treatment of nails with TA followed by urea H2O2. The data presented herein demonstrates the potential use of such PE systems with TBF in the topical treatment of onychomycosis. Whilst the concentrations of the PEs in the present study were moderately high, further studies will clarify the minimum concentrations required for effective permeation enhancement. The pre-treatment durations reported in this study were also considerably long (20 h) but again it is anticipated that future optimisation and formulation of the PEs will lead to lower pre-treatment times which will be more suitable for clinical settings and hence patient treatment.

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