A Novel Vehicle for Enhanced Drug Delivery Across the Human Nail for the Treatment of Onychomycosis

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ABSTRACT

The aim of this study was to use *in vitro* nail models to investigate the potential of a novel base formulation (Recura) containing either fluconazole or miconazole for the treatment of onychomycosis in comparison to two commercial comparators (Jublia and a Penlac generic). Initially, a modified Franz cell was used, where sections of human nail served as the barrier through which drug penetrated into an agar-filled chamber infected with dermatophytes. A second study was performed using a novel infected nail model where dermatophytes grew into human nail and adenosine triphosphate levels were used as biological marker for antimicrobial activity. The novel enhancing system Recura increased the permeation of both existing drugs through human nail sections mounted in a modified Franz cell. Furthermore, the infected nail model also confirmed that the system also enhanced the permeation through infected nail resulting in a decrease in adenosine triphosphate levels superior ($P \leq 0.05$) to Penlac generic and equivalent ($P > 0.05$) to the commercial comparator Jublia. This study demonstrated that with the use of a novel permeation-enhancing formulation base, Recura enhances delivery of miconazole and fluconazole when applied ungually such that the efficacy was equivalent or superior to commercial comparators. Such a topically applied system has the possibility of overcoming the systemic side effects of antifungals when taken orally.

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INTRODUCTION

There are reported to be up to 5 million different species of fungi that exist on Earth,[1,2] of which only a few hundred are regarded as pathogenic to humans (most frequently *Trichophyton rubrum* [*T. rubrum*], *Trichophyton interdigitale,* and *Trichophyton mentagrophytes*)[3] and are responsible for causing 50% of all nail disorders; the infection known as onychomycosis.[4] Other organisms such as yeasts (*Candida* spp.) and molds (*Scytalidium* spp., *Scopulariopsis* spp., *Fusarium* spp., *Acremonium* spp., and *Onychocola Canadensis*)[3] are also associated with onychomycosis; although predominantly 85% of all reported cases are the result of dermatophyte infections, with *T. rubrum* being the most common.[5-9] Onychomycosis is generally considered an irritant; however, it still affects around 5% to 10% of the population in western countries[6-11] and can be painful and lead to permanent disfiguration.[12] Many cases of onychomycosis are left untreated as the condition is non-life threatening; although, that said, the condition can take up to a year to treat even if the patient decides to use systemic or topical treatments.[13] Furthermore, even though the human immune system is responsible for resilience towards microorganisms,[14] it should be noted that without treatment, an onychomycosis infection will not spontaneously resolve. The treatment of onychomycosis is not trivial; and thus due to low permeability of the drug through the nail and issues surrounding re-infection from the environment; such as carpets, shoes, and socks harboring the fungal spores, the recurrence is thought to be 50% even after the patient follows the recommended treatment procedures.[12,15]

The most common type of onychomycosis is known as distal and lateral subungual onychomycosis (DLSO). DLSO generally begins at the lateral edge of the nail in a crack or fissure and spreads progressively proximally down the nail bed producing hyperkeratosis and thus onycholysis. On a day-to-day basis humans are exposed to lots of pathogenic challenges, and in particular pathogenic fungi. It is not uncommon for one toenail to show symptoms of onychomycosis with the adjacent toenails showing no symptoms at all, despite all of the toenails being exposed to the same pathogenic organisms. Thus, there is a common perception that it is damage to the nail through cracks and fissures that is critical for the disease to take hold and that without this, standard hygiene procedures are often sufficient to prevent infection. Once an onychomycosis infection has established, it is ultimately not until the underside of the nail is involved, that thickening of the nail occurs making treatment extremely difficult, considering that at this point the nail may become friable,

crumbles, and also lifts away from the nail bed[12] making systemic treatment ineffective or severely hampered. In addition, once the fungus does infect the underside of the nail, it may also proliferate in the space created by the lifting of the nail plate (known as a dermatophytoma)[16] and is often the cause of treatment failure due to the organism ultimately occupying an air space which a drug cannot permeate. Typical oral therapies for the treatment of onychomycosis include older therapies such as griseofulvin (Grisactin) and ketoconazole (Nizoral), or newer therapies such as the triazole and allylamine antifungal drugs fluconazole (Diflucan); itraconazole (Sporanox), and terbinafine (Lamisil). However, despite all of the therapies containing potent antifungals, the cure rates are low,[17,18] primarily due to poor compliance of therapeutic use by patients, high re-infection rates, and the way the organism presents itself in the infection as previously discussed. Despite poor compliance with the use of oral treatments for onychomycosis, other disadvantages of this form of treatment include systemic toxicity (e.g., nephrotoxicity and acute infusion-related reactions, such as pulmonary reactions) and drug interactions.[19,20] Thus, to avoid such issues, topical therapies such as the commercial products Loceryl (containing amorolfine) and Penlac (containing ciclopirox)[21-25] have been used frequently in the past with newer products such as Kerydin (containing tavaborole) and Jublia (containing efinaconazole) becoming more popular. However, despite topical treatments for onychomycosis being the preferred option, there is once again poor cure rates, which again are not only the result of environmental issues, long treatment times, and poor patient compliance as previously discussed, but are also the result of poor drug penetration from these products through the nail; with long treatment times (up to 12 months for toenail infections)[13,18,26] leading to poor patient compliance. For example, a clinical trial performed by the U.S. Food and Drug Administration over 52 weeks with Jublia only demonstrated complete cure of onychomycosis in 17.8%[27] of the patients treated. Thus, there is a need for better topical treatments for onychomycosis to be developed, both in terms of drug potency (low concentrations of drug, to show high therapeutic effect) and in using novel topical treatments (penetration enhancers and formulation effects) to help deliver higher therapeutic levels of drug across the nail much faster in order to decrease the time required to treat the infection.

Despite the need for a better understanding of nail drug delivery in order to achieve these therapeutic drug levels; the knowledge of nail drug delivery is relatively sparse when compared to that of the skin drug delivery over the last 60 years.[28-36] As such, it is thought that the poor therapeutic results observed with many of the topical therapies available on the market are a consequence of formulation development based on skin drug delivery, without taking into account the physical and chemical differences between the two biological matrices (nail and skin). Therefore, with a growing understanding of nail drug delivery, many of the current topical formulations are indeed considering the physicochemical properties of the drug molecule (e.g., size, shape, charge, Log *P*), the formulation characteristics (e.g., vehicle, pH, drug concentration), and the inclusion of possible penetration enhancers[37] that could aid to enhance, specifically, drug delivery through nail. Penetration enhancers such as mercaptan compounds, sulfites and bisulfites, keratolytic agents, and surfactants have all been investigated for improved drug delivery,[38,39] with a range of success rates, in that agents which permanently damaged the nail were found to be more effective than the milder treatments with reversible effects. Traynor et al[37] demonstrated that the use of novel permeation enhancing systems involving reducing and oxidizing agents, which reversibly alter the chemical structure of the nail, are feasible and not only enhance the efficacy of the existing topical formulations but also enable the delivery and efficacy of antifungals when applied topically. Further work in this area by Khengar et al[39] showed that a range of reducing and oxidizing agents using a nail swelling model could predict the enhancing effect of such agents whilst Brown et al[40] showed that treatment of the nail with thioglycolic acid followed by urea hydrogen peroxide (a combination product of urea and hydrogen peroxide) had the greatest penetration-enhancing effect on a range of model permeants.

Another issue associated with developing effective treatments for onychomycosis is the lack of availability of robust and reliable methods for testing the new or improved formulations, which take into consideration both the nail barrier and the differences in nail barrier structure following a fungal infection.[41] However, two such *in vitro* cell models for testing formulation performance are the TurChub test cell model and the onychomycosis infected nail model. The TurChub assay uses a modified Franz cell, in which sections of human nail serve as the barrier through which the drug initially penetrates prior to reaching an agar-filled receptor chamber where the dermatophytes (such as *T. rubrum*) grow. The cells are dosed with the test formulation on the surface of the nail and then incubated for a set period of time, at a set temperature. After incubation, the presence of any zone of inhibition (ZOI) is measured. The onychomycosis nail model uses human nails infected on the underside with organism (such as *T. rubrum*) mounted in the gasket section of a modified Franz cell (ChubTur cells). The nails are dosed on the dorsal side (surface) and incubated for a set time period and temperature. After incubation, the viability of the organism is measured using a validated bioluminescence assay for the presence of adenosine triphosphate (ATP). This current study used these models to investigate the potential of a novel base formulation (Recura) containing either fluconazole or miconazole for the treatment of onychomycosis in comparison to two commercial comparators (Jublia and a Penlac generic).

MATERIALS AND METHODS

MEDIA AND REAGENTS

Preparation of Sabouraud Dextrose Agar and Potato Dextrose Agar

Sabouraud dextrose powdered agar (SDA) was purchased from Remel (United Kingdom) and potato dextrose powdered agar (PDA) was purchased from Oxoid (United Kingdom). Using a measuring

cylinder, 1 L of deionized water (18.2 Mff) was transferred individually into two 1-L Duran bottles. The media was prepared by weighing the appropriate powder(s) and transferring the powders (SDA or PDA) to the individual Duran bottles containing the deionized water. Powdered SDA (65.0 g ± 0.3 g) was weighed and transferred into one Duran bottle and powdered PDA (39.0 g ± 0.3 g) was weighed and transferred into the second Duran bottle. The agar solutions were then sterilized in an autoclave for 15 minutes at 123°C ± 2°C. Once the agar had cooled to 50°C, aliquots were transferred to the appropriate vessels (90-mm Petri dishes, 25-mm square dishes, or TurChub cells). Agar aliquots were transferred into 90-mm Petri dishes (25 mL) and 245-mm square dishes (300 mL) under a laminar airflow cabinet. The Petri and square dishes containing the agar were left to solidify under the laminar airflow cabinet with the covers slightly ajar (ca. 1 cm).

Preparation of Roswell Park Memorial Institute Medium

Roswell Park Memorial Institute (RPMI) 1640 reagent (Product No. R6504) and 3-(N-morpholino propanesulfonic acid) (MOPS) were both purchased from Sigma (United Kingdom). Using a suitably sized measuring cylinder, 1 L of deionized water (18.2 Mff) was transferred into a 1-L Duran bottle. MOPS (34.53 g ± 0.3 g) was then added to the 1-L Duran bottle containing the deionized water. An aliquot of the MOPS buffer solution (900 mL) was then transferred into a 1-L Duran bottle using a suitably sized measuring cylinder. The following were then added to the 1-L Duran bottle: 10.4 g ± 0.3 g of RPMI-1640 reagent, 18.0 g ± 0.3 g of glucose (final concentration 2 g/100 mL, supplied by Sigma, United Kingdom) and 15.0 g ± 0.3 g of Agar Bacteriological (Agar No. 1, supplied by Oxoid). The solution was mixed by inversion until visually homogenous. The pH of the solution was measured using the Mettler Toledo S220 pH/Ion Meter. The solution was adjusted to pH 6.9 to 7.1 by adding 1M NaOH (prepared by adding 4.0 g of NaOH (purchased from Merck, United Kingdom) to 100 mL of deionized water (18.2 Mff)) as necessary. The solution was transferred into a measuring cylinder and additional MOPS buffer was added to make a final volume of 1 L. The solution was sterilized in an autoclave for 15 minutes at 123°C ± 2°C and then allowed to cool by equilibrating the solution in a 50°C oven for a minimum of 1 hour. Once the medium had cooled to ca. 50°C, aliquots were transferred to the appropriate vessels (245-mm square dishes or TurChub cells) and left to solidify under the laminar airflow cabinet with the covers slightly ajar (ca. 1 cm). The square dishes containing the RPMI agar were then left to solidify with the covers slightly ajar (ca. 1 cm).

Preparation of Ringer's Solution

Ringer's solution tablets were purchased from Oxoid. A total of four Ringer's solution tablets were added to a 500-mL volumetric flask. The volumetric flask was made up to 3/4 volume with deionized water (18.2 MΩ) and 250-µL Tween 80 (purchased from Acros Organics, Belgium) was added to the volumetric flask. The volumetric flask was made to volume with deionized water, and a magnetic follower was added to the solution and stirred until complete dissolution of the tablets was observed. The solution was then sterilized in an autoclave for 15 minutes at 123°C ± 2°C. The solution was allowed to cool at ambient temperature for 1 hour before storing at 2°C to 8°C until required (up to 1 month).

FORMULATIONS

A total of three formulations were supplied by Humco Pharmaceuticals (Austin, Texas) containing a novel base (Recura, US 8, 333, 981 B2) formulation comprising of the following:

- Acetylcysteine
- Alcohol
- Camphor
- EDTA
- Eucalyptus Oil
- Hydroxypropylcellulose
- Hydroxypropyl Starch Phosphate
- Magnesium Aluminum Silicate
- Menthol
- Propylene Carbonate
- Propylene Glycol
- Purified Water
- Sodium Hydroxide
- Sodium Thioglycolate
- Strontium Chloride
- Tea Tree Oil
- Thymol
- Urea

One of the formulations was a placebo comprising of the base formulation only, and the other two formulations contained two generic antifungals (fluconazole at 10% w/w and miconazole at 10% w/w). A Penlac generic (8% w/w ciclopirox topical solution) and Jublia (efinaconazole at 10% w/w) were also obtained as commercial comparators.

PREPARATION OF *T. RUBRUM* SUSPENSION

An internal reference slope culture of *T. rubrum* on SDA that was isolated from a patient suffering from onychomycosis was used in this study. The organism was subsequently sub-cultured onto fresh SDA slopes, and reference samples were placed into a glycerol solution and cryogenically frozen. Isolates of the dermatophytes were also transferred into Ringer's solution and onto PDA and incubated at 25°C for seven days using a previously tested procedure to produce conidia. The fungal colonies were then covered with 5 mL of Ringer's solution and suspensions of conidial and hyphal fragments were made by gently probing the surface with the tip of a Pasteur pipette. The spore suspension was then filtered through sterile gauze (Propax, 7.5 cm × 7.5 cm 8-ply gauze swab, BP Type 13; Smith & Nephew) to remove mycelium. The density of the suspension was assessed using a UV spectrophotometer at 600 nm, and the spore suspension adjusted until a spore count of approximately 1×10^7 cfu/mL was achieved by diluting with Ringer's solution. A serial dilution of the final spore suspension and plate count was also carried out for confirmation. The identity of the isolated strain was verified by microscopy and culture on agar.

TURCHUB ASSAY

Preparation of Human Nail Clippings (Distal)

Distal nail clippings were obtained from volunteers' toenails with informed consent and ethical approval (EC/2012/28/MedPharm),

which had been grown to a minimum length of 3 mm. Prior to acceptance of the nails into the study, all nail donors were required to have not used nail varnish or polish on their toenails within 6 months, there was no visible signs of damage and had no visible signs of disease to their nails within six months. All volunteers were asked to remove the distal nail sections using either scissors or standard nail clippers. The nail clippings were placed into an 8-mL bijou bottle per donor/donation and labelled with any details supplied and stored at 2°C to 8°C until required.

Preparation of 3-mm × 3-mm Distal Nail Segments

Using scissors, the nail clippings were cut into pieces, which were a minimum of 3 mm × 3 mm. The nail clippings were initially placed into water and heated to 60°C for 15 minutes followed by immersing in a 70% v/v ethanol in water solution and vortex mixed for one minute at ambient room temperature. The ethanol solution was then carefully decanted and replaced with a fresh 70% v/v ethanol solution and vortex mixed for a further minute. The ethanol solution was then decanted and replaced with Ringer's solution, vortex mixed for 1 minute, and decanted and replaced with fresh Ringer's solution. This process of washing with Ringer's solution was carried out a total of three times, replacing the wash solution at each phase. Once the washing process was complete, the nail clippings were placed into a sterile Petri dish without a lid and air dried under a laminar airflow cabinet for 30 minutes at room temperature. The thickness of all the nail sections was measured using a pair of calipers which had been wiped completely with a 70% ethanol-in-water solution and left to dry under a laminar airflow hood for 30 minutes immediately prior to use. Each nail section was placed into a single well of a sterile 96-well plate using forceps which had been wiped completely with the 70% ethanol-in-water solution. The lid of the 96-well plate was replaced on the plate, and the plate stored at 2°C to 8°C until used in the TurChub ZOI investigation or the infected nail investigations.

Preparation of TurChub Cells

Nail clippings (approximate size: 3 mm × 3 mm) were mounted into the validated TurChub gasket system, to ensure no leakage of formulation around the nail. The gasket system was then mounted into the TurChub cell and clamped in place between the donor and receiver compartments. The receiver compartment of each TurChub was then filled with a pre-determined calibrated volume of agar ensuring complete contact with the agar in the receiver compartment and the nail.

TurChub Zone of Inhibition Investigation

The method was performed as described in Traynor et al,[37] in which the surface of the nail mounted in the gasket section of a TurChub cell was dosed with 100 µL of test sample, and the TurChub cell was occluded and incubated at 20°C to 25°C. The *T. rubrum* organism suspension was pipetted onto the agar surface within individual TurChub cells and then left to dry under the laminar airflow hood before incubation. Infected and non-infected controls were also included in which the agar was inoculated with organism but not dosed with test sample for the infected control, and the non-infected control was not inoculated with test organism or dosed with test sample. The efficacy of the formulations was determined by measuring the ZOI of growth of *T. rubrum* in the TurChub cell. Statistical analysis of all the data was conducted using the statistical package for social science (SPSS) version 16.0 (SPSS Inc.). A one-way ANOVA and post-hoc test (Tukey) was used to compare the mean percentage ATP recovery (compared to the infected control) of each test formulations. Statistically significant differences were assumed at the 95% confidence level (i.e., when $P < 0.05$).

ONYCHOMYCOSIS INFECTED NAIL INVESTIGATION

Adenosine Triphosphate Assay Calibration

ATP calibration standards of known concentrations (220.0, 110.0, 55.0, 27.5, 13.3, 11.0, 6.9, 3.4, 1.1 ng/mL) were prepared by diluting the stock ATP standard (1 mg/mL) in Ringer's solution. These standards (100 µL) were then placed into a sterile Nunc 96-well white micro-titre plate followed by the addition of 100 µL of the MedPharm lysing agent and Promega BacTiter-Glo assay reagent. The solution was mixed for a period of 30 seconds, and the total amount of light that was emitted from the well measured every 10 seconds over a total period of 10 minutes using a Biotek FLx800 micro-titre fluorimeter/luminometer. The average of the relative light units measured over the 10 minutes was then calculated.

Preparation of ChubTur Cells

The method was performed as described in Traynor et al,[37] in which distal nail clippings of approximately 3 mm x 3 mm infected with organism were mounted into the validated ChubTur gasket system to ensure no leakage of formulation around the nail. The gasket system was then mounted into the ChubTur cell and clamped in place between the donor and receiver compartments. The receiver compartment of each ChubTur was then partially filled with an inert sterile, humidity-control medium. The cells were then incubated to allow full growth of the organism on the nail.

Infected Nail Investigation

After establishing the growth of the organisms on the nails, the nails were dosed daily for 7 days with 2 µL of the test formulations. The performance of each formulation was determined after removing the nail sample from the cell (24 hours after the final dose) and measuring the presence of viable microorganisms using a previously validated bioluminescence ATP method. Sacrificial samples were set up as an infected control to monitor the growth of the *T. rubrum* on the nail samples over the incubation period, and control nails without any infection were also set up and incubated to ensure no contamination. Statistical analysis of all the data was conducted using the SPSS version 16.0. A one-way ANOVA and post-hoc test

(Tukey) was used to compare the mean percentage ATP recovery (compared to the infected control) of each test formulations. Statistically significant differences were assumed at the 95% confidence level (i.e., when P <0.05). The relative absorption (RA) was calculated using the following equation:

$$RA(\%ATP/g/mL)^{-1} = \frac{1}{\%ATP\ MIC}$$

RESULTS

TURCHUB ZONE OF INHIBITION INVESTIGATION

A set of preliminary investigations were performed with the test formulations in Petri dishes and TurChub cells filled with SDA, PDA, and RPMI medium in order to optimize the test parameters for the TurChub ZOI investigation against the test organism *T. rubrum*. Following those preliminary investigations, the parameters for the TurChub ZOI investigation were selected, in which the nail mounted in the TurChub cell was dosed with 100 µL (infinite dose) of test sample and the TurChub cells were filled with PDA as the growth medium for *T. rubrum*. The results of this investigation are a measure of ZOI (no growth) of the organism (*T. rubrum*) on the agar surface, in which no growth is indicative of permeation of the API across the healthy nail and absorption into the agar, at concentrations of API in the agar at the site of no growth, which is equal to or higher than the potency (MIC) of the API towards the particular test organism (*T. rubrum*). Following application of a single dose (100 µL) of the base formulations containing 10% w/w miconazole or 10% w/w fluconazole for 14 days, the mean ZOI of *T. rubrum* observed were 3.57 ± 0.30 and 3.48 ± 0.15 cm, respectively (Figure 1). The mean ZOI for the cells dosed with 100 µL Jublia was 3.58 ± 0.20. There were no ZOI present after dosing of the nails with the 100 µL of the base formulation (vehicle) and the Penlac generic. The results obtained for the Penlac generic were consistent with those previously reported by Traynor et al,[37] demonstrating the reproducibility and reliability of the TurChub model as well as verifying the results obtained in this present study. The observed ZOI for Jublia, and the base formulations containing either 10% w/w miconazole or 10% w/w fluconazole were equivalent to total kill (3 cm to 4 cm) of *T. rubrum* in the TurChub cells, which is indicative of potency and high/therapeutic levels of drug permeating through the nail. It was not possible to perform a statistical comparison on all of the data as total kill was observed for 3 of the formulations; however, the base formulations with miconazole and fluconazole as well as Jublia, were all statistically (P <0.05) superior to the Penlac generic.

ONYCHOMYCOSIS INFECTED NAIL INVESTIGATION

Adenosine Triphosphate Assay Calibration

The ATP calibration assay covered the range of recovery of ATP (1.1 ng/mL to 220.0 ng/mL) from the *T. rubrum* infected nail experiment (Figure 2). The amount of luminescence measured was directly proportional to the ATP concentration; for which the level of ATP detected is an indication of the viability of *T. rubrum* in the onychomycosis nail model. This investigation was intended to demonstrate that the amount of luminescence measured directly from the nails in the infected nail study was directly proportional in terms of linearity with the amount of ATP present in the sample, which the data in Figure 2, clearly shows (R^2 = 0.9993).

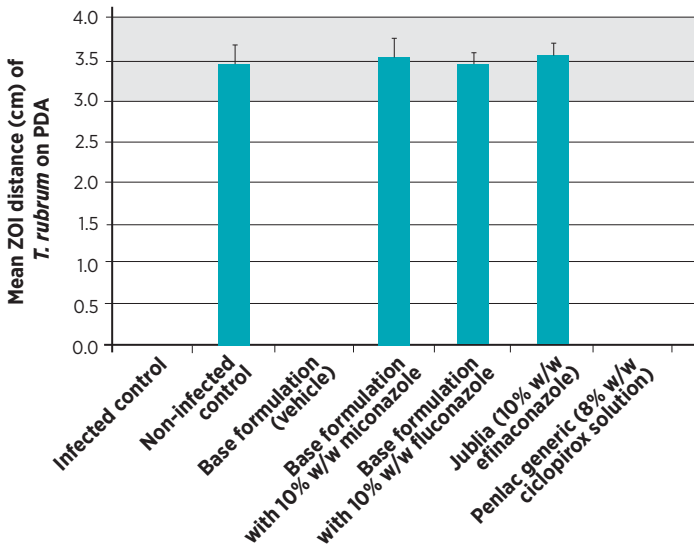
Quenching Effect Investigation

To ensure the test formulations were compatible with the ATP assay; the direct effect of the formulations on the ATP assay itself were investigated (independent of the nails and organisms). There was no substantial interference with any of the formulations (Jublia, Penlac generic, 10% miconazole in base formulation, 10% w/w fluconazole in base formulation, and the base formulation itself) with the ATP assay, in which the percentage recovery was within ± 10% of the ATP standard. Therefore, the ATP assay was found to be "fit for purpose" for the quantification of samples in the infected nail investigation, with no adjustment of values obtained required in the infected nail study.

Infected Nail Investigation

Following a preliminary infected nail experiment to optimize the experimental parameters, a daily dosing regimen of treatment of the

FIGURE 1. Full-scale ZOI assay mean distance of *T. rubrum* on PDA in TurChub cells mounted with distal nail clippings following incubation at 20°C to 25°C for 14 days, initially treated with a single 100-µL dose of appropriate test sample at *t*=0 and then inoculated with *T. rubrum* after 7 days (›mean ± SD, *n*=6 active, *n*=3 placebo). The horizontal grey bar represents the maximum range of length of agar (3 cm to 4 cm) within the TurChub cells.



nail clippings with 2 µL of the test sample daily for 7 days was selected for the infected nail investigation. The treated nails were assayed for ATP recovery following incubation at 20°C to 25°C for 24 hours after the final dose (Figure 3). The results of this investigation are a measurement of the levels of ATP in the infected nail from viable organisms (*T. rubrum*), in which a decrease in ATP in the treated nails compared to the infected control nails is indicative, that sufficient levels of API have permeated across the nail to the site of the organism infection on the underside of the nail, that is equal to or greater than the potency of the API and thus demonstrating efficacy (decrease in ATP levels). The data showed the greatest reduction in ATP recovery compared to the infected control following treatment using the base formulation with 10% w/w miconazole, the base formulation with 10% w/w fluconazole and Jublia (4.75, 6.57, and 3.33% ATP recovery, respectively), where these levels could be considered baseline (equivalent to total kill). There was no statistical difference between the percentage ATP recoveries for the three aforementioned formulations ($P > 0.05$), and the results reflect those observed in the TurChub investigation, where there was complete kill of *T. rubrum* following treatment with the same three formulations. The highest (meaning the least efficacious) percentage ATP recovery compared to the infected control was observed following treatment with the Penlac generic (20.02% ATP recovery), where once again the results are in a similar order of magnitude to those observed by other authors (Traynor et al)[38] using the same formulation. A significantly ($P \leq 0.05$) higher percentage ATP recovery was observed after the application of Penlac generic compared to the base formulation with 10% w/w miconazole and Jublia; however, there was no statistical difference between the Penlac generic and the base formulation with 10% w/w fluconazole ($P > 0.05$). These results indicate that the 10% w/w micronazole and Jublia were statistically superior than Penlac generic in the treatment of *T. rubrum* infected full-thickness nails, whereas no statistical differentiation was seen between 10% w/w fluconazole and Penlac generic possibly due to the higher variability in data observed with the 10% w/w fluconazole and Penlac generic treatments. Moderate anti-fungal efficacy was observed from the vehicle base formulation (79.96% ATP recovery), which was significantly less than that of each of the active formulations ($P \leq 0.05$).

DISCUSSION

The nail's barrier properties are known to offer a significant barrier to drug permeation in turn affecting the bioavailability of medicines applied topically. To overcome this issue, several methods to improve drug delivery have been developed. One example that has received an increasing amount of attention is the use of penetration enhancers and novel formulation approaches. In the present research, the delivery of miconazole and fluconazole from a base formulation, including a novel combination of penetration enhancers, was tested *in vitro* against two comparator products using TurChub and infected nail models. The TurChub and infected nail models themselves provide efficacy data, which is a combined result of the effects of drug potency towards the test organism (e.g., *T. rubrum*) and penetration/absorption of the drug across the nail and agar for the former and just the nail for the latter.

During the TurChub investigation, the efficacy of the novel base formulation could not be differentiated from Jublia due to the total

FIGURE 2. A representative calibration curve of ATP over the nominal concentration range of 1.1 ng/mL to 220.0 ng/mL (n=5 readings per well, ± SD).

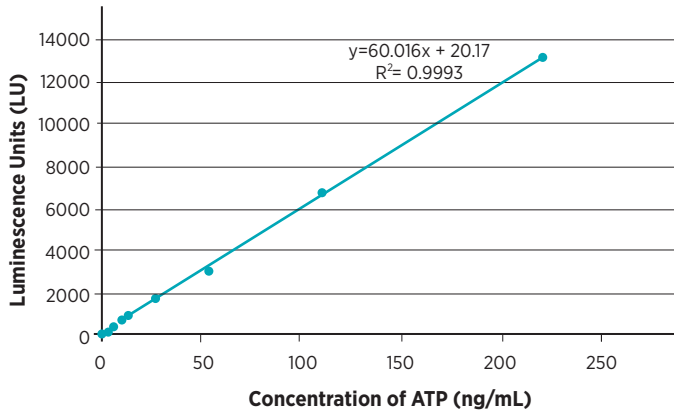
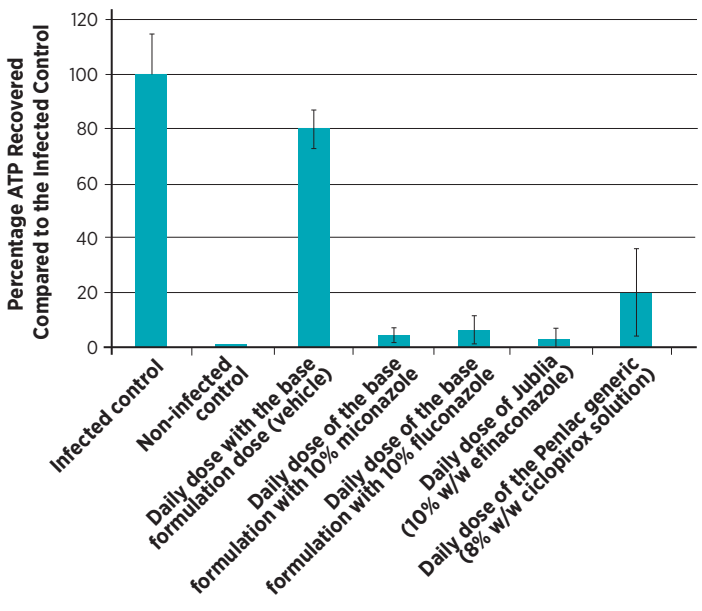


FIGURE 3. The amount of ATP recovered from the nail presented as a percentage of the *T. rubrum* infected control treated daily (2 µL) using the base formulation, the active base formulations (with 10% w/w miconazole and fluconazole), and two commercial comparators (Jublia and the Penlac generic), after incubation for 24 hours following the final treatment (mean ± SD, n=6 active, n=3 placebo/control).



kill of *T. rubrum* (Figure 1). Nevertheless, the results of TurChub test were very encouraging, indicating that the novel base formulation with either miconazole or fluconazole was equally as effective at delivering therapeutic concentrations of both drugs across full-thickness, healthy human nails. In addition, the system with each individual active (miconazole and fluconazole) was superior to the Penlac generic under the conditions tested in the TurChub model. A similar trend in results was also observed for the infected nail data, in that comparable levels of efficacy were observed with the novel base formulation (miconazole and fluconazole) and Jublia, whereas the Penlac generic was less effective than all of those formulations.

The data from the TurChub study made normalization against potency and relative agar diffusion problematic because of the maximum values observed (total kill of *T. rubrum*, following the application of three out of four formulations tested). In contrast, the infected nail study allowed differentiating the effectiveness of the different formulations easier due to the quantitative data generated, thus making it possible to normalize the results by the individual potencies of the drugs investigated against *T. rubrum* (miconazole 0.031 µg/mL to 0.25 µg/mL,[42] fluconazole 1.78 µg/mL to 2.36 µg/mL,[43] efinaconazole 0.001 µg/mL to 0.015 µg/mL,[44] and ciclopirox 0.01 µg/mL to 1.0 µg/mL[44,45]). In particular, the potency values were used to convert the % ATP data in Table 1 to give an indication of relative drug absorption (RA [see the equation]) through the nail (Table 2). The ranking for relative absorption was fluconazole > miconazole > ciclopirox > efinaconazole.

When considering ungual absorption/permeation of drugs, MW[46] and Log *P*[47,48] are reported to be the most important physicochemical properties along with formulation factors such as the use of permeation enhancers and the thermodynamic activity (TA)[49] of the drug in the formulation. Under the scope of this current study, it was not possible to comment in too much detail on the TA, as the solubility of the drugs in the formulations and the exact compositions of Jublia, Penlac generic, and the base formulations were unknown to the authors. However, the fact that all systems contained volatile solvents would suggest that upon evaporation of these solvents on the surface of the nail (following dosing) lead to all systems being at maximum TA, although this would need to be confirmed.

The MW of the drugs employed in this study (ranked from smallest to largest) were as follows: ciclopirox (207.27 g/mol) < fluconazole (306.27 g/mol) < efinaconazole (348.39 g/mol) < miconazole (416.13 g/mol). However, the ranking for relative absorption was fluconazole > miconazole > ciclopirox > efinaconazole, indicating that there is no correlation between absorption/penetration and MW. Likewise, if experimental Log *P* is considered and the drugs investigated are ranked from the most hydrophilic to hydrophobic (in which hydrophilic compounds are reported to absorb/permeate human nail more than hydrophobic compounds) then the ranking is as follows: fluconazole (0.4) < ciclopirox (2.3) < efinaconazole (3.7) < miconazole (6.1), again suggesting no real correlation with the ranking of the data in Table 2.

The absence of these correlations seems to suggest that other factors may have a role in affecting the rank order for the relative absorption of the drugs though the nail. It is interesting to note that fluconazole and miconazole ranked first (fluconazole) and second (miconazole) in Table 2 for relative absorption. Considering that both these drugs were formulated in Recura, it can be hypothesized that the formulation effects of such a novel system may have had a fundamental impact in promoting the effectiveness of fluconazole and miconazole. This is perhaps not too surprising when it is considered that this base formulation contains propylene glycol, ethanol sodium thioglycolate, acetyl cysteine, and urea. For example, sodium thioglycolate and urea combinations have been shown by Traynor et al[37] to increase the permeation of antifungals through the nail, in which the thioglycolate breaks the covalent disulphide bridges of the nail keratin, and the urea is a keratolytic agent which helps debride the nail. Ethanol, on the other hand, enhances flux by altering tissue-vehicle and drug-vehicle interactions and thus enhancing the partition.[50] The volatile nature of ethanol also means that enhancement in permeation can also be attributed to evaporative induced increase in thermodynamic activity of the drug in the formulation.[51] Acetylcysteine has long been used as a mucolytic agent and has a very good safety record, and thus provides a safe option for inclusion into nail drug delivery formulations, given that it also cleaves disulphide bonds, thus disrupting the nail structure and potentially aiding in drug permeation through the nail. Propylene glycol could also potentially aid in the drug permeation through the nail by either the solvation of keratin within the nail by competition with water for the hydrogen bond binding sites and/or acting as a transport medium for the drug in the nail. It would be interesting to investigate the individual effects of these enhancers on drug absorption and to determine if their combination, as in the formulation base, is of a synergistic nature.

The moderate antifungal effect of the placebo formulation observed in the infected nail investigation is also not too surprising given that the formulation contains tea tree oil, eucalyptus, thymol, and menthol; all of which are known to have antifungal activity. Tea tree oil is a known antifungal, where activity has been demonstrated in a broad spectrum of infectious organisms, including those associated with onychomycosis. Thymol is a known antifungal, and is an essential oil which has been used to medicate bandages prior to the use of modern antibiotics. Eucalyptus oil is also a known antifungal and has been used for the treatment of athlete's foot and other fungal infections (Aborigines are reported to have used crushed eucalyptus leaves to heal wounds and fight infections.). Camphor, on its own, has little or no antifungal activity; however, when combined with menthol[52] the pair form a potent antifungal in nail, where it is thought that the camphor acts like a penetration enhancer for the menthol, which on its own is also known to inhibit the growth of fungi.

Despite the fact that in this study Penlac generic and Jublia were only applied once in comparison to the clinical setting, where they are applied daily over months with debridement, the superiority of Jublia over Penlac was demonstrated in models in which the barrier properties of both healthy (TurChub) and diseased nails (infected nail study) were considered.[3,16,41,53-56] Such data correlates well

TABLE 1. A Comparison of the Ranking of Each of the Physicochemical Properties Combined with the TurChub and Infected Nail Results Generated in this Study, in Which for the TurChub Investigation TK Represents Total Kill, and for the Infected Nail Study, Ranking Has Been Based on Statistical Analysis of the Data.

DRUG	MOLECULAR WEIGHT		LOG *P*		MIC[42-45]		TURCHUB		INFECTED NAIL	
	G/MOL	RANK	LOG *P*	RANK	µG/ML	RANK	CM	RANK	% ATP	RANK
Miconazole	416.13	4	6.1	4	0.031 - 0.25	2	TK	1	4.57	1
Fluconazole	306.27	2	0.4	1	1.78 - 2.36	4	TK	1	6.57	3
Efinaconazole	348.39	3	3.7	3	0.001 - 0.015	1	TK	1	3.33	1
Ciclopirox	207.27	1	2.3	2	0.01 - 1.0	3	0	4	20.02	3

TABLE 2. A Comparison of the Ranking of Each of the Infected Nail Results Generated in this Study Normalized Against the MIC (Potency) Values from the Literature to Provide an Indication of Relative Absorption (TurChub Investigation Data Not Compared as Total Kill Was Observed for the Majority of the Formulations, Allowing Little Comparisons to be Made).

DRUG	RELATIVE ABSORPTION* (%ATP/G/ML)$^{-1}$		RANK
	LOWER MIC	UPPER MIC	
Miconazole	0.0068	0.0547	2
Fluconazole	0.2709	0.3592	1
Efinaconazole	0.0003	0.0045	4
Ciclopirox	0.0005	0.0500	3

*Relative absorption calculated as per equation 1.

with that observed in clinical trials,[27,57] thus suggesting that the novel base formulation containing either fluconazole or miconazole may have a material clinical benefit. The broad benefit of the novel formulation over such a broad Log *P* range (0.6 to 4.1) highlights the potential utility of this system in ungual drug delivery and should be indicative of what would be expected *in vivo*.

CONCLUSION

The treatment of onychomycosis is difficult in terms of the presentation of the disease itself (in, on, or under the nail) combined with the fact that the nail itself is a tough barrier to overcome in terms of drug delivery.[3,21,37] The difficulty in treating onychomycosis is further exaggerated by poor patient compliance, long treatment times, and a high rate of re-infections.[12,13] Thus, there is a demand for faster acting, easy-to-use, higher potency formulations for future treatment of onychomycosis. Therefore, the aim of this study was to ultimately look at a novel base formulation which had been designed specifically (US 8, 333, 981 B2) for ungual treatments. For that purpose, the *in vitro* efficacy against *T. rubrum* of the novel base formulations (Recura containing either 10% w/w fluconazole or miconazole) was compared to two commercial comparators (Jublia [10% w/w efinaconazole] and an 8% ciclopirox topical solution Penlac generic). During the TurChub ZOI assay, total kill of the organism in the TurChub cell was observed following a single treatment of the nail with Jublia and the novel base formulations (containing either 10% w/w fluconazole or miconazole). In contrast, there was no efficacy observed following treatment of the nails in the TurChub cells when treated with either the base formulation or the Penlac generic under the same test conditions. As the test formulations could not be differentiated following the ZOI investigation, MedPharm's onychomycosis infected nail model was employed as a method to quantitatively distinguish the anti-fungal efficacy of the test formulations. The *in vitro* infected nail investigation indicated that the base formulations containing miconazole or fluconazole at 10% w/w had comparable efficacy against *T. rubrum* to the commercial comparator Jublia. The active-base formulation with 10% w/w miconazole performed better than 8% ciclopirox Penlac generic, whereas the 10% w/w fluconazole did not. The incorporation of multiple penetration enhancers in the base formulation composition which aid in penetration of the drugs across the nail appears to have enhanced the anti-fungal efficacy of the active formulations. These results are promising and suggest that the base formulation can help deliver anti-fungal drugs (of varying properties) across the nail, potentially improving upon current treatments for onychomycosis.

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