Research Paper

Enhancement of tioconazole ungual delivery: Combining nanocapsule formulation and nail poration approaches

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A R T I C L E   I N F O

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Nanocapsules
Tioconazole
Onychomycosis
Multiple dosing
Poration

A B S T R A C T

This work investigated the impact of formulation including in vitro release profile, repeated dosing, and nail poration on the ex vivo nail delivery performance of antifungal formulations. Chitosan coated and uncoated tioconazole-loaded nanocapsules and a nano-based film-forming vehicle were assessed via in vitro release and in vitro permeation tests using an artificial membrane and human nail clippings, respectively. The later involved single and daily dosing experiments with intact and porated nails. Additional experiments with Nile Red-loaded formulations evaluated the depth of penetration of the fluorescent marker into the nail by laser scanning confocal microscopy. The nanocapsule formulations prolonged release of tioconazole for longer than the control solutions and this ability was related to an enhanced nail penetration of the drug. Further, the new film-forming formulation delivered its drug payload more efficiently than a marketed product. Daily dosing of the formulations doubled the amount of drug recovered from the nails. Porating the nails enhanced tioconazole delivery in single dose experiments only. The depth of penetration of Nile Red into the nails clippings ranged between 90–160 μm. This research suggests that ensuring prolonged release of a drug is fundamental to develop efficacious topical nail formulations.

1. Introduction

Onychomycosis or ungual fungal infection causes approximately 50% of the diseases of the nail yet its treatment is far from satisfactory (Baran and Kaoukhov, 2005). Systemic therapies require long periods of treatment and have potential for drug interactions and side effects (Baran et al., 2008). These limitations could be alleviated by using topical therapies but unfortunately, the effectiveness of topical products to treat onychomycosis is rather limited (Crawford and Hollis, 2007). It is believed that topical therapies fail to provide and maintain antifungal therapeutic levels at the target site including the nail bed due the low permeability of actives across the nail plate (Hao and Li, 2013; Shivakumar et al., 2013). Consequently, several strategies have been explored to improve ungual drug delivery including chemical and physical enhancement (iontophoresis and lasers) methods (Delgado-Charro, 2012; Saner et al., 2014; Shivakumar et al., 2013).

In addition, most marketed nail products are lacquers based on organic solvents (Saner et al., 2014), a formulation approach with significant limitations. When these lacquers are applied onto the nail plate, the organic solvents evaporate quickly, leaving a residue of crystallized drug unable to partition into and to diffuse through the nail plate (Hao and Li, 2013). Thus, an additional strategy to improve ungual drug delivery is to formulate vehicles that prevent crystallization of the antifungal and ensure its release and delivery over long periods of time. This capability can be assessed easily through in vitro release tests (IVRT) performed with artificial membranes across which the drug is relatively permeable as, under these conditions, the overall process is primarily rate-limited by drug release from the formulation.

Nano-size formulations present advantages for topical skin delivery; they can ensure stability of actives and act as reservoirs that provide extended drug delivery (Contri et al., 2011; Firooz et al., 2015). Recently, Flores et al. (2017) prepared a series of tioconazole-loaded cationic polymeric nanocapsules based on Pullulan, a water-soluble polysaccharide with film-forming properties. The new formulations containing 1 mg/mL of the drug showed similar antifungal efficacy than Trosid®, a marketed product containing 283 mg/mL of the drug.

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Dialysis studies demonstrated the slow release of tioconazole from the new formulations but neither the in vitro release nor the nail permeation profiles of the drug where characterized and compared to those provided by a marketed product. Thus, a first aim of this work was to investigate the potential relationship between the in vitro release (IVRT) and the ex vivo delivery performance (IVPT) for the nanocapsule formulations and the commercial product above. More specifically, this work investigated whether the antifungal performance of the new formulations (Flores et al., 2017) observed could be explained, at least partially, by their ability to provide extended release as compared to the marketed lacquer.

In contrast with the skin, nails lack the appendages and furrows where nanostructures typically localize after application of topical products (Alvarez-Román et al., 2004). Thus, it has been proposed that nails could be porated mechanically to provide sequestration sites for nanoparticles. Chiu et al., (2015) used a skin poration device (Dermroller’) to introduce a nanoparticle-based formulation into the nails. Sustained release of Nile Red from the nanoparticles was possible and diffusion was observed deeper into the nail as compared to non-porated nails. Further corroboration was obtained using TAPP-labelled nanoparticles containing octylmethoxy cinnamate; two-photon fluorescence and stimulated Raman scattering imaging were used to assess the disposition of both the particles and the marker (Chiu et al., 2015). The results suggested that topical bioavailability of antifungals could be improved by combining nail poration and formulation approaches. Thus, a second aim of this work was to test this novel approach with an antifungal drug and investigate whether tioconazole unial delivery by the new nanocapsule formulations could be enhanced further by nail poration.

To these aims, tioconazole-loaded nanocapsules suspensions were prepared and included in a film-forming formulation as previously described (Flores et al., 2017). The performance of these formulations and of Trosid® was characterized through in vitro release tests (IVRT) and in vitro permeation tests (IVPT). Additionally, the effect of nail poration as an adjuvant tool to improve nail delivery was explored. Another series of matching experiments using Nile Red and confocal microscopy investigated the depth and pathways of penetration of the fluorescent marker into the nail plate when delivered from the new vehicles. Finally, the use of different IVPT experimental (Franz diffusion cells versus in-line cells) and dosing (single versus multiple) approaches were tested.

2. Material and methods

2.1. Chemicals

Tioconazole (TIO) was bought from Pharma Nostra (São Paulo, Brazil) and Trosid® (Pfizer, Madrid, Spain) was purchased from a community pharmacy. The polymers Poli(ε-caprolactone) (MW = 70,000–90,000) and chitosan were acquired from Sigma-Aldrich (São Paulo, Brazil). Nile Red (NR) analytical grade was from Sigma-Aldrich (Gillingham, United Kingdom), Lipoid® S75 from Lipid Ingredients & Technologies (São Paulo, Brazil) and Polysorbate 80, medium chain triglycerides and cetostearyl alcohol were from Delaware (Porto Alegre, Brazil). Pullulan was kindly donated by Dinaco (Rio de Janeiro, Brazil) and Trosiderm® (Porto Alegre, Brazil). Pullulan was kindly donated by Dinaco (Rio de Janeiro, Brazil). Methanol was supplied from Tedia (São Paulo, Brazil) and methanol HPLC grade was from VWR Pro-Labo, France. All other chemicals and solvents including ethanol were of pharmaceutical grade and were used as received.

2.2. Nail clippings

The collection of human nail clippings was approved by REACH or ethics committee for the School for Health of the University of Bath (REACH; EP 11/12 115). Nail clippings at least 8 mm long were donated by healthy volunteers after providing written informed consent. The nail clippings were washed with deionized water, dried with absorbent paper and frozen (-20 °C) until use. The thickness of the nail clippings ranged between 350–490 μm. Micropores were done using a dermaroller (Infinitive Beauty™, Birmingham, United Kingdom) with 250 μm long titanium needles, which was applied on the dorsal surface of the nail by rolling it back and forth a total of 10 times.

2.3. Preparation of formulations

Aqueous suspensions containing tioconazole-loaded polymeric nanocapsules (TIO-NC) and tioconazole-loaded cationic polymeric nanocapsules (TIO-C-NC) were prepared by interfacial deposition of preformed polymer (Fessi et al., 1989) as previously described (Flores et al., 2017). Briefly, an organic phase containing 100 mg of Poly(ε-caprolactone), 10 mg of tioconazole, 0.33 mL of medium chain triglycerides and 25 mL of acetone was magnetically stirred at 40 °C for one hour. Next, 5 mL of an ethanolic solution of Lipoid® S75 (6 mg/mL) were added. This organic phase was injected into an aqueous phase (50 mL) containing 76.8 mg of polysorbate 80. The mixture was submitted to evaporation under reduced pressure and to a final volume of 10 mL; the final concentration of drug was 1.0 mg/mL. In the case of the TIO-C-NC the final volume was adjusted to 9 mL and then, 1 mL of a chitosan aqueous solution (1% of chitosan in 0.5% glacial acetic acid) was added to the mixture. The lacquer, film forming formulation (L-TIO-C-NC) was prepared by adding under magnetic stirring 9 mL of the TIO-C-NC formulation to 1 mL of an ethanol solution containing 100 mg of cetosteareth 25 and pullulan. Finally, 100 mg of pullulan were added to the mixture. The final volume was adjusted through evaporation so that the drug concentration would be 1 mg/mL.

Nile Red loaded nanocapsules (NR-NC) and cationic polymeric nanocapsules (NR-C-NC) were prepared replacing tioconazole (logP = 5.3; MW = 387.7 Da; pKa = 6.5) for Nile Red (logP = 5.0; MW = 318.3 Da; pKa = 4.8). The preparation method above was followed but the organic phase contained NR (1.5 mg) instead of tioconazole and the final concentration of marker in both (NR-C-NC and NR-NC) formulations was 0.15 mg/mL. In the case of the NR-C-NC the final volume was adjusted to 9 mL and then, 1 mL of the chitosan solution was added. The formulations containing Nile Red were filtered (0.45 μm nylon filters, Whatman, Maidstone, UK).

2.4. Characterization of tioconazole and Nile Red loaded formulations

The mean particle size and the polydispersity index were determined (n = 3) by photon correlation spectroscopy (ZetasizerNano ZS, Malvern Instruments, United Kingdom) after dilution (× 500) of each formulation with purified water. The zeta potential of the TIO-NC and TIO-C-NC formulations was determined (n = 3) from the electrophoretic mobility of particles (ZetasizerNano ZS, Malvern Instruments, UK) after sample dilution (× 500) in 10 mM NaCl previously filtered (0.45 μm nylon filters, Whatman, Maidstone, UK).

Tioconazole content was assessed by HPLC as previously described (Flores et al., 2017). Briefly, 100 μL of the TIO-NC, TIO-C-NC and L-TIO-C-NC formulations were diluted in 10 mL methanol and the mixture ultra-sonicated for 30 min. The samples were filtered and 20 μL of the filtrate was analysed for tioconazole content by HPLC (Agilent, 1260) following a method previously validated (Härter et al., 2004). The concentration of NR in the nanocapsules (NR-NC and NR-C-NC) suspension was measured by HPLC-with fluorescence detection (Dionex, Sunnyvale, CA); the excitation and emission wavelengths were 559 nm and 630 nm, respectively. The method (Chiu et al., 2015) used a HiQ sil C18W 250 × 4.6 mm (KYA Tech, Tokyo, Japan) column a methanol:water (95:5) mobile phase, a flow rate of 1 mL/min, and a 20 μL injection volume.

Encapsulation efficiency was analysed by the ultrafiltration-centrifugation technique as previously described (Flores et al., 2017). Briefly, 300 μL of either the TIO-NC or TIO-C-NC formulations were
submitted to ultrafiltration-centrifugation (Ultracel®, 10K membrane regenerated cellulose Millipore, Germany) at 5000 rpm for 7 min at 25 °C to separate the unassociated (free) tioconazole from the nano-capsules. The ultra-filtration was analysed by liquid chromatography using the same method described above.

2.5. Tioconazole and Nile Red control formulations

A series of control formulations were prepared: TIO-S was a 50:50 ethanol:water control solution containing 1 mg/mL tioconazole. A film-forming control formulation without nanocapsules (L-TIO) was prepared by adding 9 mL of TIO-S to 1 mL of ethanol containing 0.1 g of cetoesteryl and final addition of 0.1 g of pullulan to the mixture. The commercial formulation Trosid® nail solution containing 28% (283 mg/mL) of tioconazole and the excipients undecylenic acid and ethyl acetate (BNF, 2016, Electronic Medicines Compendium, 2016) was used as a comparator in multiple dose IVPT studies. Finally, a 0.15 mg/mL Nile Red solution in 50:50 v/v ethanol:water was used also as a control (NR-S).

2.6. Tioconazole and Nile Red in vitro release tests (IVRT)

IVRT (n = 3) were performed using vertical Franz diffusion cells (3.14 cm²; PermeGear Inc., Bethlehem, PA) and a silicone membrane (75 µm thick, Dow-Corning, Coventry, UK). 0.5 mL of TIO-NC, TIO-C-NC and the control TIO-S were applied resulting in a 0.16 mg/cm² normalized dose of tioconazole. 100 µL of the formulations L-TIO-NC, L-TIO and 3 mg Trosid® were applied corresponding to 0.032 mg/cm² and 0.26 mg/cm² normalized doses, respectively. The donor chamber was covered with Parafilm® in all cases. The receptor solution (~10 mL) was phosphate buffer saline (pH 7.4) with 0.5% of poly-sorbate 80 and was stirred. The whole ensemble was incubated at 32 °C. At predetermined periods, 0.5 mL of receptor medium was sampled and replaced by fresh PBS. The amount of tioconazole in the receptor samples was quantified by HPLC.

IVRT with Nile Red formulations were conducted to characterize the release profile of the fluorescent marker from the formulations NR-NC, NR-C-NC, NR-S and L-NR-C-NC later used in IVPT and LSCM imaging. These were conducted as above but the area-normalized dose (24 μg/cm²) applied of Nile Red was lower.

2.7. Tioconazole in vitro nail permeation tests (IVPT)

Several IVPT protocols (Franz versus in-line cells; single versus multiple dosing, and intact versus porated nail) were carried out to characterize the performance of the formulations in delivering tioconazole to the nail plate. Human nail tips (at least 8 mm long) from healthy donors were used in all cases; the nail clippings were soaked in deionized water for 30 min prior the experiments, to provide them with sufficient flexibility to be fitted in the nail adapter. All experiments lasted 7 days and were performed in triplicate. In all cases; the nail tips were removed from the adaptor at the end and rinsed with deionized water. The area of the nail exposed to the formulations was cut into small pieces and tioconazole was extracted by shaking the fragments with 1 mL of methanol:water (80:20) for 7 days. The extracts were analysed for tioconazole by HPLC.

2.7.1. Intact nails-single dose-Franz cells

Intact (non-porated) nail tips were placed in a nail adapter (PermeGear Inc., Bethlehem, PA, diffusion area = 0.2 cm²) which was sandwiched between the donor and receptor compartments of vertical Franz diffusion cells. The ventral surface of the nail faced the receptor chamber which was filled with ~7.5 mL of receiver solution (pH 7.4 PBS + 0.5% of Tween 80). 1 mL of the formulations TIO-NC, TIO-C-NC and TIO-S were applied to the nail dorsal surface once, on the first day of the experiment. The equivalent normalized dose was 5 mg/cm² of tioconazole. The donor chamber was covered with Parafilm® to avoid evaporation. A sample (0.6 mL) of receptor chamber was taken every 24 h and replaced with fresh medium. The ensemble was kept at 32 °C in an incubator (Heraeus, Kendro Laboratory Products, Germany) under magnetic stirring.

2.7.2. Intact nails-single dose-In-line cells

To facilitate detection of tioconazole in the receptor, a second series of experiments with non-porated nails used in-line cells (area: 0.2 cm²; PermeGear Inc., Bethlehem, PA) provided with a smaller (~0.5 mL) receptor compartment. 0.3 mL of the TIO-NC, TIO-C-NC and TIO-S formulations were applied once at the beginning of the experiment; the normalized dose of tioconazole was 1.5 mg/cm². The donor chamber was closed with Parafilm® and the receptor medium was the same as before. Non-occluded conditions and a smaller dose (0.1 mL formulation; 0.5 mg/cm² of tioconazole) were used for the film-forming formulation (L-TIO-NC) as to mimic a practical use of the formulation. The ensemble was kept at 32 °C in an incubator (Heraeus, Kendro Laboratory Products, Germany) and shaken in a horizontal shaker (brand) for 7 days. Receptor samples (0.4 mL) were collected every 24 h and replaced by fresh medium.

2.7.3. Intact nails-daily dosing-In-line cells

This protocol used in-line cells as above but the formulations (TIO-NC, TIO-C-NC, TIO-S, L-TIO-C-NC) were applied once daily (i.e., every 24 h) for 7 days. The normalized doses of tioconazole were 1.5 mg/cm² for TIO-NC, TIO-C-NC and TIO-S and 0.5 mg/cm² for L-TIO-NC as above. In the case of Trosid®, the formulation was applied with a brush twice a day as described in the patient information leaflet. To estimate the dose applied three nail clippings were weighed before and after application of Trosid®. It was estimated that application with the brush deposited 3 mg of the lacquer (or 0.85 mg of TIO or 4.25 mg/cm², assuming a density of 1 g/mL for the product). In all cases, a cotton bud imbibed in water was passed three times over the nail sample before applying a subsequent dose to remove residues from previous doses.

2.7.4. Porated nails-single and daily dosing-In line cells

In-line cells experiments with single and daily application of the formulations TIO-NC, TIO-C-NC and L-TIO-C-NC were repeated as described above but using porated nails.

2.8. Nile Red in vitro permeation tests (IVPT)

The depth of penetration of Nile Red into intact and porated nail clippings when delivered from the formulations NR-NC, NR-C-NC, NR-S and L-NR-C-NC was investigated using LSCM (Dutet and Delgado-Charro, 2012). The experiments mirrored the Franz cells protocol described above. 0.5 mL (0.38 mg/cm² normalized dose of NR) of the formulations were applied to the dorsal nail and the donor chambers were covered with Parafilm® except when the film-forming L-NR-C-NC was used (to mimic a practical application). Three different dosing strategies were investigated: (1) single application (NR-NC, NR-NC and NR-S) to intact nails; (2) daily application (all formulations above) to intact nails and (3) daily application (all formulations above) to porated nails. At the end of the 7 days long experiments (n = 3) the nail clippings were removed from the adaptor and rinsed with deionized water. 1 rectangular piece for dorsal imaging (in which case images were taken on 3 different regions) and 3 thin slices for transversal images were cut from the area exposed to the formulation.

LSCM imaging was carried out in an LSCM 510Meta inverted laser (HeNe, 543 nm) scanning microscope (Carl Zeiss, Jena, Germany). An EC Plan-Neo 40 ×/1.3 M27 oil objective was used for the dorsal images of the nail and imaging sections were captured every 2 μm. The nail specimens for transversal imaging were placed so the cross-section was a few microns from the objective (Plan-APOCHOMATH 10 ×/0.45 M27). In the case of transversal imaging, 10 measurements per piece of nail
were done using the “specify” and “plot profile” plugins in ImageJ software (US National Institutes and Health, United States of America). Both reflectance and optical images were captured simultaneously and processed with aid of ImageJ software.

2.9. Data and statistical analysis

All experiments were carried out in triplicate and the results are expressed as mean ± standard deviation. In the case of in vitro release data, the ratios of the percentage released at two-time points \([\%R_{24h}/\%R_{2h}]\) and \([\%R_{24h}/\%R_{10h}]\) were used to compare the efficiency of the formulations in releasing tioconazole during the 2–24 h and the 10–24 h intervals, respectively. For example, a \([\%R_{24h}/\%R_{2h}] = 1\) indicated that no tioconazole was released during the first and second 24 h periods, whereas a \([\%R_{24h}/\%R_{10h}] = 2\) indicated that the amount of tioconazole released had doubled in the interval considered. In the case of IVPT data, the total amount of tioconazole recovered from each nail (TIO-Rec), the apparent concentration of tioconazole in the nail (TIO/nail) and the percentage delivered to the nail with respect to the total dose applied (DRec%) were estimated.

Comparisons among formulations were done by one-way ANOVAs followed by Bonferroni’s multiple comparison tests; the effect of poration and of multiple dosing for each formulation was assessed through t-tests. The level of statistical significance was established at p ≤ 0.05.

3. Results and discussion

3.1. Preparation and preliminary characterization of the tioconazole-loaded nanocapsule formulations

The tioconazole-loaded polymeric nanocapsule aqueous suspensions (TIO-NC, TIO-C-NC) and the lacquer, film-forming formulation (L-TIO-C-NC) more suitable for practical use, were prepared and characterized as previously described (Flores et al., 2017). Table 1 shows some properties of the formulations prepared; a complete description of the formulation can be found elsewhere (Flores et al., 2017). The average tioconazole content (0.97 ± 0.02 mg/mL) for all formulations was close to the theoretical value (1 mg/mL) and the association efficiencies were close to 100%. The stability of the formulations was demonstrated for at least 30 days. The mean particle size determined by photon correlation spectroscopy was in the nanometric range (< 200 nm) for all formations and the values of polydispersity indexes were close to 100%. The stability of the formulations was demonstrated throughout the experiment with intact and porated nails. Preliminary experiments with intact and porated nails demonstrated that the nanocapsule formulation (L-TIO-C-NC) provided the fastest release followed by the film-containing nanocapsules L-TIO-NC and TIO-C-NC.

TIO-NC
Tioconazole-loaded nanocapsules
1
-7.2 ± 1.1
0.11 ± 0.03
157 ± 5

TIO-C-NC
Tioconazole-loaded cationic nanocapsules
1
+15.7 ± 0.8
0.09 ± 0.02
166 ± 4

L-TIO-C-NC
Lacquer containing TIO-C-NC
1
NA
NA
NA

TIO-S
Ethanol:water solution with TIO
1
NA
NA
NA

L-TIO
Lacquer containing TIO-S
1
NA
NA
NA

NR-NC
Nile Red-loaded nanocapsules
0.15
-6.3 ± 0.9
0.13 ± 0.04
132 ± 6

NR-C-NC
Nile-loaded cationic nanocapsules
0.15
+13.1 ± 0.7
0.15 ± 0.05
141 ± 4

L-NR-C-NC
Lacquer containing NR-C-NC
0.15
NA
NA
NA

NR-S
Ethanol:water solution with NR
0.15
NA
NA
NA

Trosid®
Commercial lacquer
283
NA
NA
NA

Table 1 Characteristics of formulations containing tioconazole (TIO) and Nile Red (NR). NA: not applicable; ND: not determined.

3.3. In vitro permeation tests (IVPT) results

Table 2 summarizes the results of single and multiple dosing experiments with intact and porated nails. Preliminary experiments comparing Franz and in-line cells resulted in relatively similar amounts of drug recovered from the nail clippings (TIO-Rec). A higher normalized dose was applied in the case of Franz cells so the percentage of dose recovered (DR%) was greater for in-line cells. Given that the smaller receptor of in-line cells facilitated quantification, this set-up was retained for subsequent experiments.

Tioconazole was easily extracted from nail clippings at the end of all experiments but no drug was quantified in the receptor in any case. The normalized amount of drug extracted from the samples (TIO/nail; Table 2), granting it represents an average concentration across the nail, compared very favourably to minimum inhibitory concentration (MIC) values (4.6 μg/mL for C. albicans, 0.5 μg/mL for T. rubrum and 0.1 μg/mL for T. mentagrophytes) (Jevons et al., 1979).

Single dose experiments with intact nail clippings suggested that
inclusion of tioconazole in nanocapsule-based vehicles was a valuable strategy (Table 2). Despite the smaller normalized dose of drug applied with the film-forming L-TIO-C-NC, this formulation delivered the greatest amount of drug to the nail clippings (TIO-Rec) and was the most efficient in delivering its cargo (DRec%). Nail poration as a potential adjuvant enhancement technique was tested next; this strategy increased delivery of tioconazole (TIO-Rec and DRec%) although the effect was only significant (p < 0.05) for the TIO-C-NC and the L-TIO-C-NC vehicles, in which case the performance approximately doubled (Table 2). These results are consistent with those reported by Chiu et al. (2015) who showed that nail poration created “sequestration sites” for nanoparticles from which the encapsulated compounds (octyl methoxycinnamate and Nile Red) were delivered. Again, the film-forming L-TIO-C-NC provided the best results with porated nails, as nearly 7% of the cargo were recovered from the nail clippings compared to 1–1.5% delivered by the other nanocapsule suspensions.

In the experiments above, the formulations were applied at the beginning of the experiments and left on place for the complete length (7 days) of the experiments. This set-up, like most published ungual drug delivery work, provides results following a single dose application. However, this approach hardly represents the real-world scenario in which patients apply lacquers once or twice daily. In addition, information about drug penetration into the nail after repeated doses is required for establishing a dosing regimen that balances efficiency of delivery with patients’ convenience. For example, Hui et al., (2017) used a multiple dosing in vitro study to investigate whether the nail concentration of a new compound exceeded the required MIC after 14 days. Thus, matching IVPT, in which the same formulations were applied daily, were performed to investigate tioconazole delivery to the nail in a simulated practical application scenario. These studies involved an additional control, the marketed formulation, Trosid® which was applied twice daily as indicated in the patient information leaflet. Multiple dosing increased significantly (p < 0.05) the amount of tioconazole delivered by the nano-based formulations to intact nail clippings but the moderate increase (1.5–2.5 fold) observed was not proportional to the 7-fold greater cumulative dose applied (Table 2). This is shown by the significantly (p < 0.05) decreased percentage of tioconazole delivered with respect to the cumulative dose applied (DRec%). In the case of porated nails, the amount recovered after multiple and single dosing was only significantly different (p < 0.05) for the TIO-C-NC vehicle and DRec% was decreased in all cases. It is possible, therefore, that the first, single dose continued to provide TIO delivery over several days so replacement by subsequent doses offered a moderate benefit. This is indeed possible, as the formulations were far from drug depletion at the end of the experiments (Table 2) and is consistent with the small differences observed when a higher normalized dose (Franz cell set up) was used. From a practical point of view, these findings suggest that daily dosing schedules with the new formulations could encumber patient (decreasing adherence) without offering significantly improved efficacy. Instead, a more convenient schedule (i.e., every two or three days) to be established by further studies could be adopted for practical applications. Surprisingly, nail poration provided no advantage in multiple dosing experiments. Because of the in vitro set up, nail poration could only be performed before mounting the nail clippings in the diffusion cells so the effect of multiple nail poration (i.e., as part of the daily application) could not be tested. Understanding why nail poration offered no advantage beyond the first dose will require further investigation. One line of enquiry is the fate of excipients and active remaining inside the nail plate from previous doses and whether these residues could hinder subsequent permeation of the active. In addition, the poration device used in this work was developed for skin application and provided relatively shallow poration depth and potentially inadequate pore geometry (see later, Nile Red experiments).

The comparison with Trosid® provides additional insights, it was estimated (see Materials and methods) that approximately 17 times more tioconazole was applied with this formulation than with the film-forming L-TIO-C-NC. However, the marketed product delivered only ~7 times more drug to the nail than the L-TIO-C-NC lacquer. In other words, the best experimental formulation (L-TIO-C-NC) provided increased efficiency of delivery (DRec%) but delivered less drug (TIO-Rec) to the nail plate than the marketed product (Table 2). Whether this indicates the need for further optimization of the new formulations is debatable as the apparent concentration of antifungal in the nail (TIO/Nail values) were above the MIC in all cases (Table 2). Potentially, further development and optimization would be required to ensure that drug concentrations exceed MIC across the whole nail thickness and the
Table 2

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>TIO-NC</th>
<th>TIO-C-NC</th>
<th>TIO-S</th>
<th>L-TIO-C-NC</th>
<th>Trosid®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single dose</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Franz In tact</td>
<td>D</td>
<td>5</td>
<td>S</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>TIO/NC</td>
<td>151 ± 58</td>
<td>348 ± 101</td>
<td>108 ± 42</td>
<td>108 ± 42</td>
<td>108 ± 42</td>
</tr>
<tr>
<td>DRec%</td>
<td>0.15 ± 0.06</td>
<td>0.35 ± 0.10</td>
<td>0.12 ± 0.04</td>
<td>0.12 ± 0.04</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>In-line intact</td>
<td>D</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>TIO/Rec</td>
<td>2.9 ± 0.4</td>
<td>2.6 ± 0.09</td>
<td>1.8 ± 0.2</td>
<td>1.8 ± 0.2</td>
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<tr>
<td>DRec%</td>
<td>0.06 ± 0.12</td>
<td>0.87 ± 0.03</td>
<td>0.59 ± 0.08</td>
<td>0.59 ± 0.08</td>
<td>0.59 ± 0.08</td>
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<tr>
<td>In-line Porated</td>
<td>D</td>
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<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>TIO/Rec</td>
<td>3.5 ± 0.3</td>
<td>4.3 ± 1.0</td>
<td>6.9 ± 0.1</td>
<td>6.9 ± 0.1</td>
<td>6.9 ± 0.1</td>
</tr>
<tr>
<td>DRec%</td>
<td>1.16 ± 0.10</td>
<td>1.42 ± 0.33</td>
<td>6.89 ± 0.12</td>
<td>6.89 ± 0.12</td>
<td>6.89 ± 0.12</td>
</tr>
<tr>
<td>Daily dosing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-line intact</td>
<td>D x N</td>
<td>1.5 ± 7</td>
<td>1.5 ± 7</td>
<td>0.5 ± 7</td>
<td>4.25 × 14</td>
</tr>
<tr>
<td>TIO/Rec</td>
<td>4.6 ± 0.1</td>
<td>6.2 ± 1.7</td>
<td>8.8 ± 2.8</td>
<td>8.8 ± 2.8</td>
<td>8.8 ± 2.8</td>
</tr>
<tr>
<td>DRec%</td>
<td>0.22 ± 2×10⁻³</td>
<td>0.30 ± 0.08</td>
<td>1.25 ± 0.40</td>
<td>1.25 ± 0.40</td>
<td>1.25 ± 0.40</td>
</tr>
<tr>
<td>In-line Porated</td>
<td>D x N</td>
<td>1.5 ± 7</td>
<td>1.5 ± 7</td>
<td>0.5 ± 7</td>
<td>4.25 × 14</td>
</tr>
<tr>
<td>TIO/Rec</td>
<td>5.3 ± 1.3</td>
<td>7.7 ± 0.6</td>
<td>5.1 ± 0.11</td>
<td>5.1 ± 0.11</td>
<td>5.1 ± 0.11</td>
</tr>
<tr>
<td>DRec%</td>
<td>0.25 ± 0.06</td>
<td>0.37 ± 0.03</td>
<td>1.18 ± 0.45</td>
<td>1.18 ± 0.45</td>
<td>1.18 ± 0.45</td>
</tr>
</tbody>
</table>

IVPT results (mean ± SD, n = 3) for the different set-ups tested. D (mg/cm²): Area-normalized dose of tioconazole applied; N: number of doses applied in multiple dosing experiments, TIO-Rec (μg/g) and TIO/nail (μg/g) absolute and normalized tioconazole recovered from nail samples at the end of experiments, DRec%: percentage recovery with respect to total (cumulative) dose applied. Significant (p < 0.05) effect of: Franz versus in-line cells, ℓporated versus intact nail, *multiple versus single dose, in matching experiments. Superscripts |α–| identify pairs of values that are significantly (p < 0.05) different.

3.4. In vitro permeation experiments with Nile Red formulations

The lipophilic marker, Nile Red, replaced tioconazole in a series of formulations (NR-NC, NR-NC and L-NR-NC) used for LSCM imaging. NR content across the formulations was 0.14 ± 0.01 mg/mL very close to values expected. All the formulations released NR over 72 h with L-NR-NC and NR-NC providing the slowest release, releasing around 40% and 70% at the end of experiment time, respectively.

The distribution of NR throughout the nail was investigated after single and multiple dose nail permeation experiments. The x-y planar LSCM images in Fig. 3 illustrate the preferential localization of the marker at the periphery of the onychocytes, suggesting the intercellular pathway to be the favoured route of permeation for Nile Red into the nail. This is consistent with previous observations (Chiu et al., 2015) and with the larger lipid content in this the region of the nail plate (Kobayashi et al., 1999). Fig. 3B corresponds to a porated nail clipping and the intense fluorescence illustrates clearly one of the fissures created which acted, as expected, as reservoir for the formulations.

Transversal images of non-porated nails following multiple dosing with several formulations are available in the supplementary material (Fig. S1). The depth of penetration of NR was measured in triplicate and...
10 measurements were taken per replicate. The depth of penetration for the marker was similar for single and daily applications and unexpectedly, shallower for mechanically porated nails (Table 3). Overall, these results are consistent with the IVPT data generated for tioconazole (Table 2). The depth of Nile Red penetration ranged from 90 to 160 μm across all experiments suggesting that the marker had reached only the upper dorsal region of the nail plate. Assuming these results can be extrapolated to tioconazole, this would explain why no drug was found in the receptor in IVPT. This data signals the need for new experimental methods that provide the concentration profile of antifungal drugs across the whole nail plate in order to predict in vivo performance of formulations and guide rational development.

Hui et al. (2002, 2017) used a nail sapling instrument based on a drill that provides the average concentration of the active in the “dorsal/intermediate” and the “ventral/intermediate” regions of the nail plate. Finally, Fig. 3B shows that the fissures created by the dermaroller were rather elongated. Chiu et al. (2015) could visualize these “elongated cracks” at least as deep as 32 μm into the nails. It could be argued that a purpose-made nail poration device that formed narrower but deeper pores would be more efficient in enhancing delivery.

4. Conclusion

The new nanocapsule-based formulations provided extended release of tioconazole across synthetic membranes and this in vitro release capacity was related to their ex vivo performance as ungual drug delivery systems and potentially, their antifungal activity previously reported. The new film-forming formulation provided the best efficiency of delivery ex vivo and delivered a higher percentage of its drug payload than a marketed product. One mechanical nail poration with a skin device increased tioconazole delivery to nails in single, but not in multiple dose experiments indicating the need for an improved nail poration strategy. Tioconazole nail delivery was approximately doubled by daily application of the formulations. Experiments with the fluorescent marker Nile Red supported the observations with tioconazole and suggested a penetration depth of 90–160 μm attained after 7 days exposure. Overall, the results suggested that judicious combination of formulation and nail poration approaches provide a valuable approach to improve the performance of ungual drug delivery products. However, further optimization of the formulations here presented and of the nail poration strategy are required.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at [doi](https://doi.org/10.1016/j.ijpharm.2017.11.008)

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