In Vitro Penetration of A Novel Oxaborole Antifungal (AN2690) into the Human Nail Plate

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ABSTRACT: Onychomycosis is a challenging fungal infection to treat topically, likely due to the unique properties of the nail plate. This seemingly impenetrable barrier has high resistance to the passage of antifungal drugs in sufficient concentrations to kill the causative fungi deep in the nail bed. Recently, a new class of antifungal agent was described, termed oxaboroles, which have broad-spectrum activity. These oxaboroles were designed with properties believed to be required to allow for easier transit through the nail plate. Herein, we report (i) the nail penetration results of four oxaboroles that led to the selection of AN2690, (ii) the results of the nail penetration of AN2690 from four vehicles, and (iii) the nail penetration of AN2690 in its chosen vehicle compared to a commercial control, ciclopirox. AN2690 has superior penetration compared to ciclopirox, and achieves levels within and under the nail plate that suggest it has the potential to be an effective topical treatment for onychomycosis. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: absorption; solubility; log p; drug effects; diffusion; onychomycosis; AN2690; nail penetration

INTRODUCTION

Onychomycosis is a common and complex fungal infection, affecting 13–14% of the US population.[1,2] In diabetic and elderly patients, the incidents range from 33% to 50%.[3,4] Onychomycosis is typically caused by dermatophytes.[1,5,6]

Onychomycosis treatment is usually difficult. Systemic therapy, including oral terbinafine and oral itraconazole, requires a relatively long-term treatment that may cause side effects and frequently has a high level of recurrence. An attractive alternative is topical application of an antifungal directly to the nail.[7] However, the only topical treatment approved for use against onychomycosis in the USA is ciclopirox (see Tab. 1), formulated as Penlac®. Current topical treatments have poor clinical efficacy due to the nail's unique properties such as its thickness and compact construction, and the infection's deep-seated nature. Consequently, most antifungal drugs that successfully treat skin fungal infection do not effectively penetrate throughout the nail plate and nail bed. To achieve an effective chemical concentration into and through the human nail plate, a successful local therapy is dependent upon an appropriate antifungal drug that overcomes the nail's unique barrier behavior.

In a recent report, a new class of boron containing compounds, called oxaboroles, was described that demonstrated broad-spectrum

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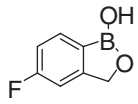
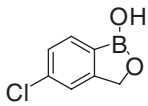
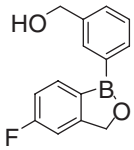
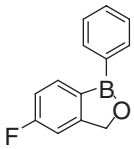
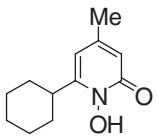
Table 1. Penetration Results of Four Oxaboroles into the Nail Plate, Compared to Their Physicochemical Parameters

Compound	Structure	MIC[a,b,8] (μg/mL)	Physicochemical Parameters[b] MW	cLogP	Absolute Amount in the Nail Plate After Single Dose Left for 3 Days[c] Ventral/Intermediate Layer (μg/mg nail)	Dorsal/Intermediate Layer (μg/mg nail)
AN2690[d]		1	152.0	1.24	2.47 ± 3.79	2.07 ± 0.77
1		1	168.4	1.81	0.78 ± 0.63	2.27 ± 1.66
2		1	242.0	2.51	0.43 ± 0.67	2.01 ± 0.96
3		4	212.0	3.55	0.00 ± 0.00	1.91 ± 1.60
Ciclopirox[e]		0.5	207.0	2.03	0.00 ± 0.00	1.47 ± 0.34

[a,b]Minimum inhibitory concentration against *T. rubrum*.
[b]Data provided by Anacor Pharmaceuticals, Inc.
[c]Each number represents the mean ± SD of three samples.
[d]Test article was formulated at 10% w/v (100 g/L) in ethanol.
[e]Test article was formulated at 8% w/w (80 g/kg) in commercial lacquer.

antifungal activity against yeast, molds, and dermatophytes. Further evaluation of the antifungal ability of this new oxaborole family concluded that 5-fluoro-1,3-dihydro-1-hydroxy-2,1-benzoxaborole (AN2690) was the most effective, especially against the dermatophytes *T. rubrum* and *T. mentagrophytes*, fungal pathogens that are the primary cause of onychomycosis.[8]

The aim of this study was threefold: first, to select the one oxaborole that showed the best ability to penetrate the nail plate from a panel of four candidates; second, to couple this oxaborole with a delivery vehicle that should maximize it's delivery into the nail bed; third, to compare the *in vitro* nail penetration efficacy of the final oxaborole/vehicle combination to the topical onychomycosis drug, ciclopirox, formulated in its commercial nail lacquer.

MATERIALS AND METHODS

Test Article and Dosage Formulation

AN2690 (5-fluoro-1,3-dihydro-1-hydroxy-2,1-benzoxaborole), 1 (5-chloro-1,3-dihydro-1-hydroxy-2,1-benzoxaborole), 2 (5-fluoro-1,3-dihydro-1-(3-hydroxymethylphenyl)-2,1-benzoxaborole), and 3

(5-fluoro-1,3-dihydro-1- phenyl-2,1-benzoxaborole) were obtained from Anacor Pharmaceuticals, Inc. (Palo Alto, CA). [^{14}C]-AN2690 was synthesized by Amersham Biosciences UK Limited (Buckinghamshire HP7 9NA, UK) radiochemical purity and specific activity of >99.3% and 55 mCi/mmol, respectively.

Penlac® nail lacquer (ciclopirox 8% topical solution) was manufactured by Dermik (Berwyn, PA). [^{14}C]-Ciclopirox (pyridinone-6-(^{14}C)-ciclopirox) was synthesized by Perkin-Elmer Life and Analytical Sciences (Boston, MA). The radiochemical purity and specific activity of the chemical was >95% and 12.5 mCi/mmol, respectively.

Nail Penetration Procedure

Details of the nail penetration study have been previously described.[9,10] Briefly, a cadaver finger nail plate with no obvious signs of infection or damage was mounted in a one-chamber diffusion cell (Fig. 1; Permegear, Inc., Hellertown, PA) with the outer dorsal surface (top center) open to the air and the inner ventral surface in contact with a small cotton ball acting as a supporting nail bed. The supporting cotton ball under the nail was wetted with normal saline to provide moisture to the nail plate, and the degree of hydration was monitored and controlled during the experiment. The mounted nails were incubated at 32°C and $44 \pm 8\%$ relative humidity starting 24 h prior to the first dose, to allow the nail plate to stabilize to the approximate natural hydration level, and for the duration of the experiment. The exposed dosing area was 0.78 cm^2 and aliquots (10 μL) were applied to this surface once daily.

Dosed surface area washing was conducted at the end of the experiment for the single dose study, or each morning before the next dose for the multiple dose study. The dosed surface area of the nail was washed with cotton tips in a cycle, as follows: two times with ethanol, then with 50% Ivory® liquid soap (Procter & Gamble, Cincinnati, OH), then two times with distilled water. The washing samples from each cycle were pooled. Under the controlled humidity and temperature, we did not observe any abnormal situations such as the nail plate color change, hydration changes, or fungal growth during the 14-day dosing period. After completion of the experiment, the nail plate was transferred to a cutting holder for sampling. The nail plate was secured in position so that the outer dorsal-dosed surface faced the holder and the inner ventral layer faced the cutting drill. The cutting holder was moved to bring the ventral surface just barely in contact with the cutter tip. The drill was then started and a fine adjustment moved the mounted nail plate into the cutter tip, removing a powder sample from the layers of the nail plate. In this way, a core approximately 0.3–0.4 mm in depth and 7.9 mm in diameter was drilled from the lower layers of each nail, and the powder was harvested. These powder samples are referred to as the "ventral/intermediate section" (Fig. 1). The dosed section of nail plate above the powder sampling area was cut out as one piece and saved as "the dorsal/intermediate section" (Fig. 1). The remaining nail plate outside of the dosing area was then saved as the "remainder nail plate" (Fig. 1). All the nail plate samples were individually collected into a glass scintillation vial and weighed.

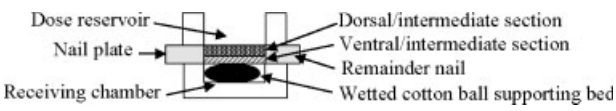


Figure 1. Cross-section of one-cell diffusion chamber showing sections of nail sampled.

Pilot Study 1: Nail Penetration of Four Oxaborole Compounds

AN2690, 1, 2, and 3 (Tab. 1), formulated at 10% (w/v) in ethanol, were compared for their ability to penetrate the nail plate. A single aliquot (10 μL) of each formulation was dosed to the dorsal layer of the human nail plates using the nail penetration procedure described above. After 3 days, the dosed area was washed and the dorsal/intermediate and ventral/intermediate nail samples were collected, stored at 4°C and analyzed for drug using LC/MS/MS.

Pilot Study 2: Effect of Vehicle on AN2690 Nail Penetration

Four formulations, all containing 10% (w/w) AN2690 were compared for their ability to deliver AN2690 to the deep layers of the nail plate and into the nail bed. Formulation A: 70% ethanol, 20% poly (vinyl methyl ether alt maleic acid monobutyl ester) (w/w); formulation B: 56% ethanol, 14% water, 15% poly (2-hydroxyethyl methacrylate), 5% dibutyl sebacate (w/w); formulation C: 55% ethanol, 15% ethyl acetate, 15% poly (vinyl acetate), 5% dibutyl sebacate (w/w); formulation D: 20% propylene glycol, 70% ethanol (w/w). Using

the nail penetration procedure described above, aliquots (10 μL) of the dose formulations were applied to human nail plates once daily for 14 days. The cotton ball supporting bed was collected from each cell chamber and replaced with a new one at days 5, 10, and 15 after the first dose. The ventral/intermediate nail samples were collected at the end of the 14-day dose period, stored at 4°C and analyzed for drug by LC/MS/MS.

Pivotal Study: Nail Penetration of AN2690 Compared to a Commercial Control

AN2690, 10% (w/v) in propylene glycol/ethanol (1:4, v/v) was compared to ciclopirox, 8% (w/w) in commercial nail lacquer were for their ability to penetrate nails. Trace amounts of carbon-14 radiolabeled AN2690 and ciclopirox were added to their respective formulations the day before the first dose. Using the nail penetration procedure described below, aliquots (10 μL) of the dose formulations were applied to human nail plates once daily for 14 days. The cotton ball bed supporting the nail was collected from each cell chamber and replaced with a new one every 72 h after the first dose (days 3, 6, 9, 12, and 15). The dorsal/intermediate, ventral/intermediate and remaining nail samples were collected at the 14-day dose period. The radioactivity of all samples, including the washings, were analyzed and compared.

Quantitative Analysis of Oxaboroles from Pilot Studies 1 and 2

LC/MS/MS (API3000, Applied Biosystems, Foster City, CA) was used to quantitate the amounts of nonradiolabeled oxaboroles, AN2690, 1, 2, and 3 in samples from the nail penetration studies. For the cotton ball analysis eleven calibration standards were prepared fresh in normal saline. A volume of 100 μL of each standard was spiked onto a fresh cotton ball with final calibration standard concentrations of 0, 2.5, 5, 10, 20, 40, 80, 160, 320, 640, 1280, and 2560 μg/mL. Acetonitrile (Burdick & Jackson, Muskegon, MI) containing the internal standard *p*-nitrophenol (PNP) was added to all cotton balls. The cotton ball samples and any residual solvent were transferred to centrifuge filter tubes. After centrifugation, the filtrate from the cotton ball samples was transferred to autosampler vials and analyzed by LC/MS/MS. For the ciclopirox samples, the filtrate was first derivatized with dimethylsulfate according to a previously described method[11] before analysis by LC/MS/MS. Samples with calculated concentrations above the highest calibration standard were diluted 10- or 20-fold with acetonitrile containing internal standard PNP (TCI America, Portland, OR). For the nail analysis, two separate calibration curves were prepared, one for nail powder analysis and one for top of the nail analysis. Each curve contained eleven calibration standards. Standards were first prepared in dimethylsulfoxide. A volume of 10 μL of each standard was spiked onto keratin powder (TCI, Tokyo Kasei Kogyo, Tokyo, Japan) (6.5 mg for nail powder curve and 17 mg for top of the nail curve). Nail samples were digested with 1 N NaOH overnight at 45°C. The next morning, before extraction with methylenechloride, the pH of the samples was adjusted to pH 3. After extraction, the organic layer was transferred and evaporated. Samples were reconstituted in acetonitrile and analyzed by LC/MS/MS using an Eclipse XDB-C18 5 μm, 2.1 mm × 50 mm column (Agilent, Wilmington, DE) and a gradient mobile phase from 5 mM ammonium acetate and acetonitrile. The extraction efficiency reported for ciclopirox from hoof membranes was approximately 80%.[11] The extraction efficacy of AN2690 recovered from spiked nail clippings was determined to be $89 \pm 2.7\%$ ($n = 3$).

Radioactivity Measurement from the Pivotal Study

All radioactivity measurements were conducted with a Model 1500 Liquid Scintillation Counter (Packard Instrument Company, Downer Grove, IL). The counter was audited for accuracy using sealed samples of quenched and unquenched standards as detailed by the instrument manual. The ^{14}C counting efficiency is equal to or greater than 95%. All nail samples pretreated with Packard soluene-350 were incubated at 40°C for 48 h followed by the addition of 10 mL scintillation cocktail (HIONIC-FLUOR, Packard Instrument Company, Meriden, CT). Other samples (standard dose, surface washing, and bedding material) were mixed directly with Universal ES scintillation cocktail (ICN Biomedicals, Costa Mesa, CA). Background control and test samples were counted for radioactivity for 3 min each.

Calculations and Data Analysis

Quantitation of nonradioactive compounds was based on peak area ratios of compound to internal

standard. The method of regression for the calibration curves was selected based on the best fit. Linear and quadratic regression was used with $1/x$ or $1/x$ squared weighting. All integrations were performed using Analyst version 1.3 (Applied Biosystems). The concentrations of compound in the cotton balls were converted to absolute amounts by taking the sample volume of 100 μL into account. The amount of compound in the nail powder from the inner ventral/intermediate section and dorsal/intermediate section of the nail were adjusted for their respective weights and reported in μg/mg nail.

The individual and mean (±SD) amount of test chemical equivalent in nail, bedding material, and wash samples are presented as dpm, μCi, percent administered dose, and mg equivalent at each time point. The concentrations of [^{14}C]-labeled test chemicals were calculated from the value based on the specific activity of each [^{14}C]-labeled test chemical. The information of concentration of nonlabeled test chemical in the topical formulation was obtained from the manufacturers. The total concentration of test chemical equivalent is the sum of the concentration of [^{14}C]-labeled test chemical and the concentration of nonlabeled test chemical. The value of the total amount of test chemical equivalent in each nail sample was calculated from those values based on the radioactivity of the sample and the ratio of total mg test chemical equivalent and radioactivity of the test chemical. The data was further normalized by dividing by the weight of the sample.

Terminology

Ventral/Intermediate Center

Powdered nail sample drilled from the center of the inner surface (facing the nail bed) approximately 0.3–0.5 mm in depth to the surface. The area is beneath the dosed site of the nail plate but does not include dosed surface (dorsal nail surface).

Dorsal/Intermediate Center

Nail sample cut from the center of the outer surface and immediate area of dosed site approximately 0.3–0.5 mm in depth. The area is beneath the dosed site of the nail plate and includes the dosed surface.

Remainder Nail

The nail plate outside of the dosed area that remains after the ventral/intermediate section and dorsal/intermediate section have been removed.

Supporting Bed

The cotton ball placed within the Teflon chamber of the diffusion cell to support the nail plate, provide moisture, and receive chemicals penetrating through the nail plate.

Surfacing Washing

Ethanol (or other organic solvents) and soap/water washing on the surface of the dosed site.

Ring

A plastic ring placed on the top of the nail plate to prevent leakage from the dose site onto rest of the nail plate or inside of the cell chamber.

Cell Washing

Ethanol (or other organic solvents) and soap/water wash of the inside of the diffusion cell.

RESULTS

In the first pilot study, the nail permeation capacity of four oxaboroles, AN2690, AN1677, AN2718, and AN2416, each 10% (w/v) in ethanol was compared. The chemical deposition of each compound in the dorsal/intermediate layer center and the ventral/intermediate layer center of the nail plate, and the relevant chemical parameters (MW and $c\log p$) are summarized in Table 1. Seventy-two hours after a single dose application, the 1-hydroxy oxaborole derivatives AN2690 and AN2718 penetrated to the lower ventral/intermediate layer to a greater extent than the 1-phenyl derivatives AN2416 and AN1677. AN2690 showed the greatest nail penetration and was selected for the subsequent studies.

The second pilot study was used to select the optimal vehicle for AN2690 from four vehicles, including three nail lacquer formulations. The vehicle compositions, concentration of AN2690 found in the ventral/intermediate section and the amount of AN2690 found in the cotton ball supporting bed at end of the study are reported in Table 2. The results show that the penetration of AN2690 into the lower ventral/intermediate nail section and into the cotton ball supporting bed were not statistically different for these four vehicles. Because no vehicle offered a clear

Table 2. Effect of Vehicles on Nail Penetration of AN2690

Formulations, 10% w/w AN2690 (Vehicle)	AN2690 in Deeper Nail/Nail Bed After 14 Days Daily Dosing[a,b]	
	Cotton Ball Nail Supporting Bed (μg/Sample)	Ventral/Intermediate Layer Center (μg/mg Nail)
(A) (70% ethanol, 20% poly (vinyl methyl ether alt maleic acid monobutyl ester))	294 ± 92	3.62 ± 0.95
(B) (56% ethanol, 14% water, 15% poly (2-hydroxyethyl methacrylate), 5% dibutyl sebacate)	257 ± 119	4.02 ± 2.05
(C) (55% ethanol, 15% ethyl acetate, 15% poly (vinyl acetate), 5% dibutyl sebacate)	418 ± 207	5.56 ± 2.46
(D) (20% propylene glycol, 70% ethanol)	752 ± 675	8.96 ± 4.06

[a]Each number represents the mean ± SD of group ($n = 5$).
[b]No statistical significance was found between the vehicles.

penetration advantage, the simple solvent vehicle, formulation D, was selected for further development.

In the final, pivotal, study, the nail penetration of the oxaborole, AN2690, was compared to the commercial reference compound, ciclopirox. The results are listed in Tables 3–6.

The nail characteristics for both groups were evaluated. In both cases, the thickness of whole nail plate, the depth of the ventral surface core sample removed by the cutter, the percentage of the whole nail thickness, and the actual weight of powdered nail sample collected, are compared in Table 3. No statistical difference was found between the two groups.

The concentration of AN2690 or ciclopirox in each section of the nail samples are shown in Table 4. After weight normalization, the concentration of AN2690 in the dorsal/intermediate section, ventral/intermediate section, and remainder nail samples was significantly higher than that of ciclopirox ($p \leq 0.002$).

The cumulative amounts of AN2690 or ciclopirox found in the cotton ball supporting bed samples at each time point are reported in Table 5. The amount of AN2690 found in the cotton ball samples was significantly higher than that of ciclopirox ($p < 0.05$). By study end, the amount of AN2690 found in the cotton balls amounted to approximately 250 times that of ciclopirox.

The mass balance of the radiolabeled material recovered from the AN2690 and ciclopirox groups is shown in Table 6. This data summarizes radioactive recovery from washing, nail, and supporting bed cotton ball samples. There was no statistical difference found between the two groups.

DISCUSSION

The physical and chemical differences between the nail plate and stratum corneum are probably

Table 3. Quantity of Nail Samples from Ventral (Inner) Surface to Center of the Nail Plate from AN2690 and Ciclopirox Groups

Groups	Whole Nail Thickness (mm)	Nail Core Sampled from the Ventral (Inner) Surface Center of the Nail Plate[a,b]		
		Depth of Core (mm)	% Whole Nail Thickness	Powder Sample Collected (mg)
AN2690	0.68 ± 0.17	0.32 ± 0.11	47.31 ± 10.53	6.9 ± 2.7
Ciclopirox	0.65 ± 0.17	0.29 ± 0.10	44.93 ± 11.66	5.3 ± 3.8

[a]Each number represents the mean ± SD of each group ($n = 6$).
[b]No statistical significance was found between the two groups.

Table 4. Comparison of Weight Normalized AN2690 and Ciclopirox Equivalent in Each Part of Nail Plate Samples After 14-day Treatment

	Radioactivity as μg Equivalent/mg Nail Samples[a]		
Nail Samples	AN2690	Ciclopirox	*p*-Value (*t*-test)
Dorsal/Intermediate Center	25.65 ± 8.80	7.40 ± 3.47	0.0008
Ventral/Intermediate Center	20.46 ± 4.72	3.09 ± 2.07	0.0001
Remainder Nail	26.06 ± 12.41	4.38 ± 2.73	0.0022

[a]Each number represents the mean ± SD of each group ($n = 6$).

responsible for the lack of efficacy of the topical nail antifungal formulations presently on the market.[12] As a permeation barrier, the behavior of a human nail plate does not mimic human skin, though they are both rich in keratin. The human nail possesses high sulfur content (cystine) in its hard keratin domain, whereas the stratum corneum does not, and its thickness is approximately 100 times greater than the stratum corneum.[13] Walters and Flynn[14] reported that the permeability coefficients of alcohols diluted in saline through nail plates was five times greater than the permeability coefficients of neat alcohols. The nail plate is thus a hydrogel, which swells when hydrated.[15,16] In addition, the upper (dorsal) layer, even though only a few cell layers thick, constitutes the main barrier to drug diffusion into and through the nail plate. Therefore, a topically applied chemical has to penetrate into the dense, hydrophilic keratin fabric network containing fewer pores, and pass through a longer distance compared to the skin to reach the deeper layers of the nail plate and the nail bed. As a result, the concentration of an applied drug across the nail can drop about 1000-fold from the outer surface to the inner surface.[17] To complicate matters, onychomycosis, frequently in a hyperkeratotic presence, involves the deeper nail layer and the nail bed. Hence, it is difficult for a topically applied antifungal drug to achieve a therapeutically effective level in the inner ventral layer, let alone the nail bed.[18] To optimize the nail penetration of topical treatments, it is important to consider the nail's unique barrier properties and develop an antifungal drug that has matching physicochemical properties.

In this study, we set out to identify such a compound—one that is compatible with the nail plate and can penetrate to the nail bed in a therapeutic concentration. We first conducted two pilot studies, the first to select an antifungal compound that has the best physicochemical properties for nail penetration and the second was to identify a clinically relevant formulation that would maximize the penetration of the chosen antifungal agent. We then conducted a pivotal study to compare our final drug/formulation selection with the commercial reference, ciclopirox, formulated as Penlac®.

In the first pilot study, the nail permeation capacity of four oxaborole antifungals, AN2690, AN1677, AN2718, and AN2416 (Tab. 1) was determined. These compounds range in molecular weight of 152–242 Da and in $c\log p$ of 1.24–3.55. Ethanol was employed as the only vehicle to avoid variability of aqueous solutions or aqueous-organic solvent mixtures, which can cause deswelling.[19] All four compounds dissolve readily in

Table 5. Cumulative Amounts of AN2690 and Ciclopirox (mg Equivalent) in Cotton Ball Supporting Bed Samples After 14-day Treatment

	Radioactivity as mg Equivalent/Samples[a]		
Sampling Day (Time Period)	AN2690	Ciclopirox	*p*-Value (*t*-test)
Day 3 (0–72 h)	0.0609 ± 0.0605	0.0011 ± 0.0020	0.0043
Day 6 (72–144 h)	0.2160 ± 0.1875	0.0024 ± 0.0047	0.0022
Day 9 (144–216 h)	0.6052 ± 0.5413	0.0042 ± 0.0077	0.0022
Day 12 (216–288 h)	1.2826 ± 1.1752	0.0056 ± 0.0094	0.0022
Day 15 (288–360 h)	2.2405 ± 1.7325	0.0089 ± 0.0131	0.0022

[a]Each number represents the mean ± SD of each group ($n = 6$).

Table 6. Summary of Mass Balance of Radioactivity After 14-day Treatment

	Radioactivity as Percent Applied Dose (%)[a]	
Items	AN2690	Ciclopirox
Surface washing	25.30 ± 23.23	69.80 ± 11.20
Ring	33.60 ± 13.55	15.36 ± 9.82
Dorsal/intermediate center	1.77 ± 0.35	0.69 ± 0.26
Ventral/intermediate center	0.74 ± 0.34	0.09 ± 0.08
Remainder nail	14.43 ± 14.60	3.15 ± 1.23
Supporting bed	11.73 ± 8.92	0.05 ± 0.08
Inner washing	0.50 ± 0.61	0.21 ± 0.34
Total[b]	88.06 ± 9.21	89.34 ± 1.56

[a]Each number represents the mean ± SD of each group ($n = 6$).
[b]No statistical significance was found between the two groups (t-test).

ethanol at 10% (w/v) and employing this single solvent allows the penetration capacity of the test articles to be determined independently. Applying a single dose and allowing the compounds 72 h to penetrate, we found the smaller, more polar compounds AN2690 and AN2718 penetrated to the greatest extent. The larger, more lipophilic compounds did not penetrate as well. This suggests that nail permeability of the oxaborole class increases as the molecular weight and/or lipophilicity decreases (Tab. 1), although further studies are required to determine whether molecular weight or lipophiliciy has the greatest influence. From this study, we selected AN2690 for further development.

In the second pilot study, the effects of four different vehicles, containing 10% (w/w) AN2690 were examined for deep nail penetration. Nail lacquer formulations are a popular choice for topical antifungal treatment. They typically contain a film-forming agent, solvent, antifungal drug, and possibly a penetration enhancer. Once the lacquer is applied, it forms a thin film containing a supersaturated antifungal drug. This film provides a chemical gradient to drive drug flux as the drug is released.[10] We took a similar approach in the development of a vehicle for AN2690 and developed three nail lacquer vehicles, believing that this approach could further enhance the nail penetration of this compound. AN2690 dissolves readily in ethanol, without heating, at concentrations up to 50% (w/v). Therefore, ethanol was used as the base solvent for all our vehicles. We formulated AN2690 at a concentration of 10% (w/w) in these vehicles, which all contained above 50 % (w/w) ethanol. The three lacquers varied by the film-forming agent. In formulation (A) we chose a durable lacquer that could withstand bathing, the warm, moist environment of the shoe and potential damage from friction with socks, etc. In this formulation, the film forming agent, poly (vinyl methyl ether alt maleic acid monobutyl ester), leaves a water insoluble film that is very durable and resistant to damage. However, this durability makes it difficult to remove. Since we believe that residual film left on the nail plate would impair the penetration of subsequent doses, the lacquer needs to be removed prior to reapplying the drug. Therefore, in formulations B and C, we developed lacquers that could be more easily removed. In formulation B, the film forming agent, poly (2-hydroxyethyl methacrylate), forms a film that is water soluble. This can be removed by simply washing the nail plate with soap and water but it is not as durable as formulation A. In formulation C, the film forming agent, poly (vinyl acetate), leaves a water insoluble film that can be removed by peeling, or scratching the film away from the nail plate, but like formulation B, it is not as durable as formulation A. For our final formulation we decided to use a simple solvent vehicle. The ethanol vehicles used in the first pilot study had already provided good penetration; it is easily applied, dries quickly, and does not leave a residue. In preliminary studies, application of formulations A–D to cadaver nails showed no visible precipitation of the drug after the solvent had evaporated, implying that in formulations A–C, the drug was probably supersaturated within the film. In formulation D, no solid residue was observed after evaporation of the solvent either and we speculate that the drug may be supersaturated on the surface/dorsal layer of the nail plate and possibly held in solution by the natural moisture/lipid content of the nail plate itself. We tested these formulations in a nail penetration study, applying them daily for 14 days. The data from this second experiment (Tab. 2) showed that none of the four vehicles had a significant impact on the nail penetration of AN2690. Approximately the same amount of AN2690 penetrated the nail plates regardless of the vehicle. Given that penetration from the simple solvent vehicle, formulation D, was equal to the lacquers, we decided to pursue this formulation since it

provides the most dosing options for clinical development.

In the pivotal study, the *in vitro* penetration capacity of AN2690, 10% (w/v) in propylene glycol and ethanol (1:4, v/v), was compared to ciclopirox, 8% (w/w) in its commercial lacquer formulation. Ciclopirox is a synthetic hydroxypyridone with a relative low molecular weight (MW = 207.3) and lipophilicity ($c\log p = 2.03$) (Tab. 1). It is active, *in vitro*, against a broad spectrum of clinically relevant fungi, including dermatophytes, yeasts, and molds.[8] Ciclopirox has been in use as a topical antifungal agent since 1982 and has been well studied over the last two decades.[20–22] Thus, ciclopirox nail lacquer is a very useful control reference for evaluation of the potential of AN2690.

Table 4 shows the amount of AN2690 that penetrated into the lower layers of the nail plate, the ventral/intermediate section, was approximately five times higher than that of ciclopirox ($p = 0.001$). Table 5 shows that AN2690 penetrated through the nail plate and into the supporting cotton ball at levels approximately 250 times greater than ciclopirox. The overall amount of AN2690 that penetrated through the nail in the 14-day period amounted to 16% of the applied dose compared to 0.1% for ciclopirox. Considering the antifungal activities of AN2690 and ciclopirox are similar (Tab. 1), one could hypothesize that AN2690 should show significantly better clinical efficacy than the current commercial treatment. Therefore, AN2690 was selected as a clinical development candidate.

As previously reported,[23] the *in vitro* nail experiments were hydration controlled to mimic normal physiological conditions. The modified nail sampling instrument improved the ability to capture nail powder during the sampling process. It enabled well-controlled, accurate, and reproducible sampling of the inside of the nail and high mass balance efficiency (Tab. 6).

In conclusion, nail penetration studies were used to (i) identify a lead oxaborole antifungal agent for clinical development, (ii) select a suitable vehicle, and (iii) compare penetration of the final drug/vehicle to the commercial topical treatment, ciclopirox. This resulted in the selection of AN2690 in ethanol/propylene glycol (4:1) as vehicle. This product shows a superior permeation capability into and through the normal human nail plate *in vitro* compared to the commercial treatment, ciclopirox. Concentrations of AN2690 delivered in the deep nail layer and nail bed were significantly higher than the ciclopirox controls. These results suggest that AN2690 has the potential to be an effective topical treatment for onychomycosis. AN2690 is currently in clinical development for the treatment of onychomycosis and preliminary reports are showing clinical efficacy superior to previous topical trials.[24] The results of these trials will be reported in future publications.

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