In Vitro Nail Penetration of Nitric Oxide-releasing Formulations for the Topical Treatment of Onychomycosis

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ABSTRACT

Background: Onychomycosis is a common fungal infection of the nail caused primarily by dermatophytes. Topical treatments are favored over orally administered agents due to their side-effect profile and reduced risk of drug-drug interactions. However, efficacy rates for current topical therapies are hampered due to low penetration of drug across the nail plate. Nitric oxide-based topical therapies are promising due to the ability of the gaseous species to readily diffuse across the nail. Previous experiments have demonstrated the potent fungicidal effect of Novan's nitric oxide-releasing drugs in vitro. The aim of this study was to evaluate the ability of nitric oxide, released from several unique drug product formulations (gel, cream, lacquer), to penetrate the human nail and effectively kill the fungal infection. **Methods:** In vitro human nail penetration was evaluated (MedPharm, Guilford, UK) utilizing the ChubTur® infected human nail assay. *Tricophyton rubrum (T. rubrum)* was inoculated to the underside of human nails and allowed to establish infection for 14 days. Following the establishment of infection, the nails were mounted into modified Franz cells and topical treatments were applied to the top of the nail plate. The penetration of several unique nitric oxide-releasing formulations was evaluated by assessing the viability of *T. rubrum* after exposure via bioluminescence quantitation of ATP. **Results:** All nitric oxide-releasing topical formulations demonstrated effective fungal killing in 24 hrs, after a single treatment application. **Conclusions:** The mean reductions of viable fungal hyphae observed with various nitric oxide-releasing formulations ranged from 82% - 99% with several candidate formulations performing as well or better than 10% efinaconazole in this assay (83% mean reduction, Jublia®). The minimal difference in fungicidal effects between the various nitric-oxide formulations suggests that penetration of nitric oxide gas through the nail plate was sufficient to kill the fungal infection after a single treatment application. Taken together these data demonstrate that nitric oxide-releasing treatments, with rapid penetration of the nail plate and eradication of fungal infection, are comparable to Jublia (10% efinaconazole solution) and represent promising novel, topical therapies for the treatment of onychomycosis.

MATERIALS & METHODS

In Vitro Time-Kill Assays: Time-Kill assays were performed in accordance with the ASTM E2783-11 Standard. Varying concentrations of nitric oxide-releasing drug substance (NVN1000 or NV4000) were incubated with *Tricophyton rubrum* ATCC #28188 (10^6 CFU/ml) in Tris buffer (100 mM, pH 7.5-7.7) at 37°C and untreated cultures were included as controls. At 4 and 24 hours of incubation samples were taken and serial dilutions were plated on Emmon's Sabourad Dextrose Agar with product neutralizers to determine the number of viable fungi. The percent and $\log_{10}$ reductions in the fungal population of the challenge strain were determined following exposure to each concentration of drug substance at 4 and 24 hrs.

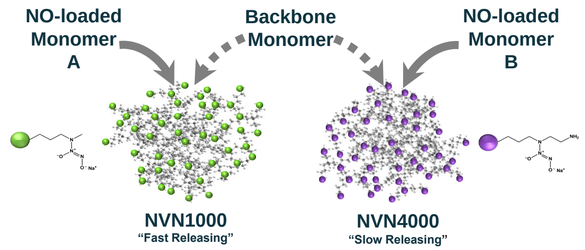
In Vitro Nitric Oxide Release Assay: The active drug phase was mixed in a 1:1 ratio with the appropriate hydrogel phase at room temperature in a custom reactor with a controlled gas flow of nitrogen to carry any nitric oxide released from the admixtures to the detecting unit. The admixture nitric oxide release profiles were determined using a custom designed apparatus (Novan) interfacing with a nitric oxide analyzing unit (Sievers). Each admixture was analyzed until no non-baseline level of nitric oxide was detected, or for 24 hours, whichever came first. The resulting nitric oxide release profiles are depicted in Figure 3a and the total nitric oxide release profile was plotted versus the square root of time (Figure 3b).

ChubTur® Infected Nail Study: Full-thickness nails were individually infected on the underside of the nail with a clinical isolate of *T. rubrum* and were subsequently mounted onto the nail gasket within the ChubTur® cell. The receiver chamber of the cell was filled with Ringer's solution and the nails were incubated at 25°C for 14 days to establish a robust infection. Following the 14 day infection period, the ChubTur® cells were removed from incubation and a dose (50 µL) of mixed test formulation was applied to the apical surface of the nail opposite of the *T. rubrum* inoculation. The nails were then incubated with test formulation at 25°C for 24 hrs. Following test formulation exposure, the nails were cleaned of residual formulation while in the gasket of the ChubTur® cells with deionized water and were subsequently wiped dry with a cotton swab to remove any moisture. Once clean the nails were removed from the ChubTur® cells and were subsequently placed in individual wells of a 96-well plate containing the ATP standard (55.0 ng/mL) and analyzed for the presence of ATP from the viable fungi via an ATP luminescence assay. ATP calibration standards of known concentrations were prepared via sequential dilution of a stock ATP standard (1 mg/mL) in Ringer's solution. The wells of the 96-well plate were analyzed for the presence of ATP and the ATP recovery was compared to ATP standards and untreated positive controls. The mean percent ATP recovery form each test formulation was compared to the infected control and statistical analysis was performed using a one-way ANOVA with a post-hoc Tukey's test using a 95% confidence interval.

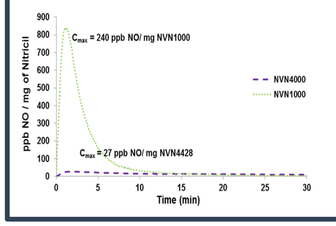
RESULTS

Figure 1. Nitric oxide-releasing Drug Candidates and In Vitro Nitric Oxide Release Profiles of Drug Substance (NVN1000 or NVN4000) in PBS (37°C, pH 7.4) A) Nitric oxide-releasing polysiloxane macromolecules can be designed with **B**) different instantaneous fluxes of nitric oxide while maintaining **C)** the same total amount of nitric oxide released over time**.**

A) Nitric Oxide-Releasing Macromolecules



B) Nitric Oxide Release Rate



C) Total Nitric Oxide

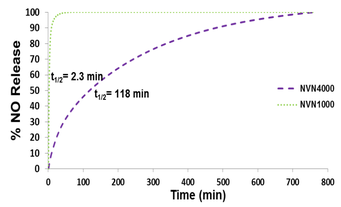
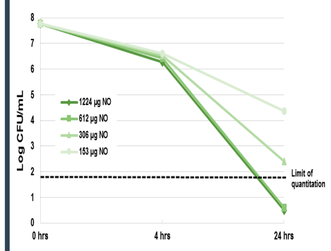


Figure 2. In Vitro *T. rubrum* Minimum Inhibitory Concentrations (MICs) and Time-Kill Assays with Nitric Oxide-Releasing Drug Substance Candidates. A)The MICs for both NVN1000 and NVN4000 were determined previously and *T. rubrum* cultures were incubated with varying concentrations of either **A**) fast-releasing NVN1000 or **B)** slow-releasing NVN4000 drug substance in 100 mM Tris buffer (pH 7.5-7.7) to assess fungicidal activity following a single exposure for either 4 or 24 hrs.

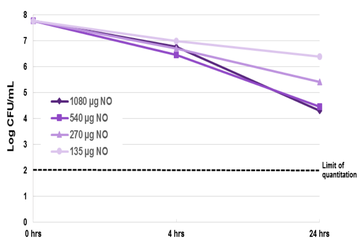
A) Minimum Inhibitory Concentrations

Drug Substance	MIC (µg NO)
NVN1000	153
NVN4000	68

B) NVN1000



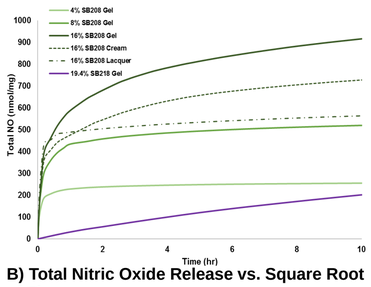
C) NVN4000



Conclusions: NVN1000 demonstrated fungicidal activity at the **same** concentration of drug substance necessary to inhibit growth (MIC = 153 µg NO) while NVN4000 fungicidal activity (3-log reduction) was observed at eight times the minimum inhibitory concentration (MIC = 135 µg NO) previously observed for *T. rubrum.*

Figure 3. In vitro Nitric Oxide Release Profiles of Drug Product Candidate (SB208/SB218) Formulations Following Admixture at 32 °C Detected via Nitric Oxide Chemiluminescence. A) The total nitric oxide release over time was assessed and **B)** the gaseous nitric oxide release rate was determined along with additional **C)** release kinetic parameters for candidate formulations.

A) Total Nitric Oxide Release from Candidate Formulations



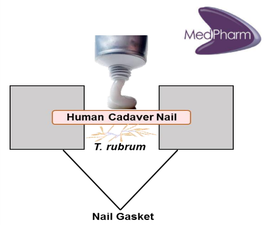
B) Total Nitric Oxide Release vs. Square Root of Time

C) Nitric Oxide Release Kinetics Parameters of Candidate Formulations

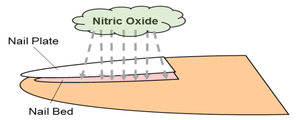
Formulation	C_{max} (nmol/mg)	Total NO, 1 hr (nmol/mg)
4% SB208 Gel	0.87	228.85
8% SB208 Gel	1.74	434.31
16% SB208 Gel	0.91	587.97
16% SB208 Cream	1.44	475.46
16% SB208 Lacquer	1.79	489.10
19.4% SB218 Gel	0.02	24.09

Figure 4. In Vitro Penetration of Various Nitric Oxide-Releasing Formulations Through the Nail Bed as Measured via Vertical Diffusion Cell. A) Schematic of ChubTur® cell utilized for infected nail study and nitric oxide penetration through the nail bed **B)** ATP release assay (mean ± SEM) following a single application of various formulations. Six nail samples (n=6) for active formulations and three nail samples (n=3) for non-infected controls for various nitric oxide-releasing formulations following once daily application to nail surface **C)** Mean percentage kill of *T. rubrum* in the ChubTur® infected nail investigation.

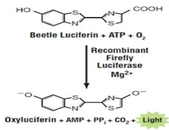
A) Schematic of ChubTur® Apparatus



Gaseous nitric oxide should readily penetrate the nail



B) ATP Release Assay and ATP Recovery from *T. rubrum* Infected Nails Following a Single Application of Various Formulations



Test Formulation	Mean ATP Recovery
non-infected control	1.06 ± 0.43
4% SB208 Gel	18.02 ± 6.40
8% SB208 Gel	11.7 ± 4.90
16% SB208 Gel	11.33 ± 4.72
16% SB208 Cream	8.38 ± 2.09
16% SB208 Lacquer	0.98 ± 0.30
19.4% SB218 Gel	16.9 ± 9.43
Jublia (10% efinaconazole soln)	17.51 ± 6.71

C) Mean Percentage Fungal Kill in the ChubTur® Infected Nail Assay

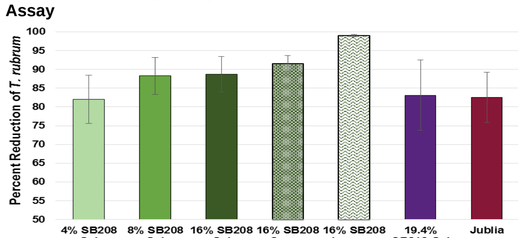
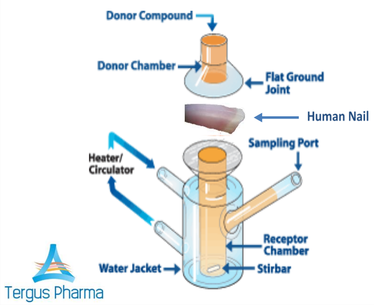
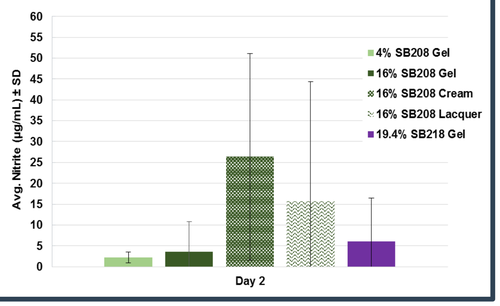


Figure 5. In Vitro Penetration of Various Nitric Oxide-Releasing Formulations Through the Nail Bed as Measured via Vertical Diffusion Cell. A) Diagram of a vertical diffusion cell and **B)** average nitrite recovered ± SD for various nitric oxide-releasing formulations following once daily application to the nail surface.

A) Schematic of a Vertical Diffusion Cell



B) Nitric Oxide Nail Penetration



CONCLUSIONS

- **In Vitro time-kill assays against *T. rubrum* demonstrated greater than >99.9% killing following 24 hrs of exposure to concentrations of either NVN1000 and NVN4000 demonstrating the ability of both nitric oxide-releasing candidates to exhibit fungicidal activity.**
  - Superior fungicidal activity was observed following exposure to NVN1000 where as low as 153 µg of NO for 24 hrs demonstrated a 3-log reduction in *T. rubrum* fungal counts while the same concentration of NVN4000 corresponded to only a 1-log reduction of *T. rubrum.*
- **Topical treatment with various nitric oxide-releasing topical formulations exhibited potent reduction in *T. rubrum* fungal viability in an *in vitro* infected nail study and all candidate formulations demonstrated comparable efficacy.**
  - Although not statistically different from one another, the 16% Lacquer formulation and the 16% Cream formulation were more efficacious in this *in vitro* infected nail model system.
  - The slow-releasing formulation (19.4% SB218 Gel) exhibited a modest reduction in antifungal activity when compared to the matched NO content fast-releasing SB208 Gel formulation – suggesting that the rapid burst of nitric oxide from the NVN1000-containing formulations might be the optimal NO release profile for superior penetration and fungicidal activity.
- **These findings suggest that the rapid bust of gaseous nitric oxide from the NVN1000-containing SB208 formulations lead to enhanced nail penetration and may provide superior efficacy in the clinical setting for the treatment of onychomycosis and other cutaneous fungal infections.3**