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Report

Efficacy of NVC-422 in the treatment of dermatophytosis caused by *Trichophyton mentagrophytes* using a guinea pig model

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Abstract

Objectives Dermatophytes, belonging to genera including *Trichophyton*, *Epidermophyton*, and *Microsporum*, are the causative agents of superficial fungal infections, prevalences of which are estimated to be as high as 25% in the worldwide population. This study evaluated the activity of topical formulations of NVC-422 (sodium 2-[dichloroamino]-2-methylpropane-1-sulfonate), the lead compound in a new class of antimicrobials that consist of broad-spectrum, fast-acting, nonantibiotic antimicrobial molecules based on the endogenously produced *N*-chlorotaurines.

Methods The antifungal efficacy of NVC-422 was investigated using a guinea pig model of infection with *Trichophyton mentagrophytes*. Infected guinea pigs were randomly assigned to four treatment and two control groups. The efficacy of the treatments was assessed clinically and mycologically at 72 hours after the final topical dose.

Results The test compound 2% NVC-422 in 1% Noveon Gel demonstrated the highest level of clinical efficacy. Outcomes of treatment with all other test compounds differed significantly from outcomes in the untreated control group ($P = 0.003$, $P = 0.029$, $P = 0.012$, and $P < 0.0001$, respectively). Fungal elements were detectable in skin sections from untreated guinea pigs but not in skin sections obtained from any of the treatment groups.

Conclusions Evaluation of the efficacy of NVC-422 in the treatment of dermatophytosis using an experimental guinea pig model showed that this compound possesses potent antifungal efficacy as measured by mycological and clinical endpoints. The highest degree of clinical and mycological efficacy was demonstrated by 2% NVC-422 in 1% Noveon Gel. These data show that NVC-422 has potent antifungal activity *in vivo*. Clinical evaluation of NVC-422 in the treatment of superficial infections caused by dermatophytes, including onychomycosis, is warranted.

Introduction

Dermatophytes, belonging to genera including *Trichophyton*, *Epidermophyton*, and *Microsporum*, are the causative agents of superficial fungal infections, all of which have worldwide distribution. The most common of these are *Tinea pedis* (athlete's foot), *Tinea capitis* (affecting the scalp), *Tinea corporis* (affecting the body), and *Onychomycosis* (affecting the nails). The prevalence of superficial fungal infections has been estimated to be as high as 25% in the worldwide population, although the distributions of clinical manifestations and causative agents vary by geographic location.[1] Similarly, it is estimated that the incidence of tinea pedis approaches one-third of the adult population in Europe,[2,3] and an epidemiological study comparing subjects with and without diabetes in the USA revealed a 40% incidence of the disease among the study population as a whole.[4] *Onychomycosis* affects 3–13% of

the population in the USA and Europe, with higher prevalences (> 30%) in older people.[5–7] The incidence of this disease is increasing as a result of a growing elderly population and the spread of human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS). Other predisposing factors leading to onychomycosis include immunosuppression, poor peripheral circulation, diabetes mellitus, nail trauma, and *tinea pedis*.[8]

The treatment of *onychomycosis* has improved considerably over the past 10 years as a result of the introduction of oral terbinafine and itraconazole.[9] However, despite the encouraging efficacy of these antifungal agents, many nail infections are notoriously difficult to treat and may require a longer duration of treatment and relapse is common. Consequently, there is a need for novel topical antifungals that are safe and effective for the treatment of hair, skin and nail (*onychomycosis*) infections.

NVC-422 (sodium 2-[dichloroamino]-2-methylpropane-1-sulfonate) is the lead compound in a new class of antimicrobials called Aganocide® compounds which are being developed by NovaBay Pharmaceuticals, Inc. (Emeryville, CA, USA). These Aganocide® compounds consist of broad-spectrum, fast-acting, nonantibiotic antimicrobial molecules based on the endogenously produced *N*-chlorotaurines.[10] Importantly, these molecules have demonstrated a low potential for developing resistance.[11] *Trichophyton mentagrophytes* is a zoophilic fungus that causes hair, skin, and nail infections. In this study, we evaluated the activity of topical formulations of NVC-422 in the treatment of dermatophytosis using a guinea pig model of infection with *T. mentagrophytes* ATCC 24953.

Materials and methods

Laboratory animals

The antifungal efficacy of NVC-422 was investigated using a guinea pig dermatophytosis model described previously.[12] Guinea pigs were chosen as test subjects because their susceptibility to dermatophytosis is similar to that of humans, and their large body surface provides sufficient area to perform experiments to determine the clinical and mycological effects of potential antifungal agents. The *in vivo* experimental protocol was approved by the Animal Care and Use Committee of the Case Western Reserve University. All procedures in the protocol were conducted in compliance with the Animal Welfare Act passed by Congress in 1966, most recently amended in 2008, the Guide for the Care and Use of Laboratory Animals (Eighth Edition, The National Academies Press), and the Office of Laboratory Welfare. According to the protocol, male albino Sprague–Dawley guinea pigs (Harlan Laboratories, Inc., San Diego, CA, USA) with a body weight of 450–500 g were housed in the Animal Resource Center, Case Western Reserve University. The environmental controls for the animal room were set to maintain a temperature of 16–22 °C, a relative humidity of 30–70%, and a 12:12-h light:dark cycle. Experimental animals underwent an acclamation period for a minimum of five days prior to use.

Fungal strain

Trichophyton mentagrophytes ATCC 24953 was chosen as the strain to challenge the guinea pigs. This particular strain was chosen because the skin infection it causes allows for the observation of redness, crusting, exudates, and hair root fungal invasion.[12] From a frozen stock, *T. mentagrophytes* was subcultured on potato dextrose agar (PDA) (Difco Laboratories, Inc., Detroit, MI, USA) plates, wrapped with parafilm (Pechiney Plastic Packaging, Inc., Menasha, WI, USA), and incubated at 30 °C for seven days. Following incubation, the conidia were collected from the surface of plates using sterile cell scrapers (BD Falcon™; BD Biosciences, Inc., Bedford, MA, USA) and sterile normal saline solution (0.85% NaCl). The conidia were washed three times in sterile normal saline and resuspended in the same solution. Ten-fold dilutions of conidial suspension were prepared and counted using a hemacytometer. A working suspension of conidia was prepared at a final concentration of 1×10^7 colony-forming units (CFUs)/100 μl in normal saline.

Animal challenge

Each guinea pig was anesthetized with a cocktail of ketamine 100 mg/ml (Ketaset®; Fort Dodge Animal Health, Fort Dodge, IA, USA), xylazine 20 mg/ml (Anased® Injection; Akorn, Inc., Decatur, IL, USA), and acepromazine 10 mg/ml (Boehringer Ingelheim Vetmedica, Inc., St Joseph, MO, USA) (3 : 3 : 1; v/v/v) administered intramuscularly. Using an electric shaver, an area to the left of the midline of the animal's back was clipped on each guinea pig. The area was then shaved with a disposable razor (Gillette™; Proctor & Gamble, Inc., Boston, MA, USA). A square measuring 2.5 × 2.5 cm was drawn on the skin. Marked areas were then evenly abraded using sterile fine grit sandpaper. A cell suspension containing 1×10^7 *T. mentagrophytes* conidia in 100 μl of sterile normal saline was applied and rubbed thoroughly on the abraded skin using a sterile pipette tip.

Antifungal therapy

Infected guinea pigs were randomly assigned to six groups to be treated with: (i) 0.5% NVC-422 in 0.5% Noveon Gel ($n = 10$); (ii) 1% NVC-422 in 1% Noveon Gel ($n = 10$); (iii) 1.5% NVC-422 in 1% Noveon Gel ($n = 10$); (iv) 2% NVC-422 in 1% Noveon Gel ($n = 10$) and (v) 1% Noveon Gel (vehicle control) ($n = 10$). The sixth group comprised infected untreated control animals ($n = 6$). The concentrations were selected based on preliminary experiments (data not shown); the final composition and viscosity was designed for application to the skin as a nondrip formulation.

Animal treatment commenced 72 hours postinoculation and continued three times per day for seven days. Treated animals received 0.2 ml of the drug applied topically to the infected area using a sterile pipette tip. The vehicle-treated control guinea pigs received 1% Noveon Gel. Infected untreated control guinea pigs received no treatment. Figure 1 shows the study design.

Clinical and mycological evaluation

The efficacy of the treatments was assessed clinically and mycologically at 72 hours after the final topical dose (study day 13). Clinical efficacy was based on a visual assessment and preceded mycological assessment.

The infected area marked on the back of each animal was divided into four quadrants. Clinical assessment of the infected skin was performed by scoring the skin in each quadrant on a scale of 0–5 as follows: 0 = no lesions; 1 = a few slightly erythematous sites on the skin; 2 = well-defined redness and swelling with bristling hairs; 3 = large areas of marked redness, encrustation, scaling, bald patches and ulceration in places; 4 = partial damage to the integument and loss of hair; and 5 = extensive damage to the integument and hair loss. Scores for the quadrants were summed for each animal and used to determine the clinical efficacy of the respective treatments. The efficacy of each treatment was expressed as a percentage derived from the score of the treatment group relative to the score of the infected untreated control group using the following formula:

$$\text{Percentage efficacy} = 100 - (T \times 100/K)$$

where T = the test group score and K = the infected untreated control group score. The score for any treatment group signifies the average of clinical scores obtained for animals in the same group.

The hair root invasion test was used to assess the mycological cure rate resulting from treatment with the test articles.[13] Hair samples were removed (after the clinical assessment was completed) with sterile forceps from the four quadrants on the backs of the guinea pigs. Ten uprooted hairs from each quadrant were planted onto the surface of PDA plates divided into corresponding quadrants. Plates were incubated at 30 °C for 2–4 days. Following incubation, the number of hairs exhibiting fungal growth at the hair root was counted using a stereomicroscope (Fig. 2). Counts from the quadrants were summed for each animal and used to determine the mycological efficacy of the respective treatments. The effectiveness of a compound in reducing the number of fungus-positive hair samples was expressed as a percentage derived from the number of fungus-positive hair samples in the treatment group relative to the number of fungus-positive hair samples in the infected untreated group using the following formula:

$$\text{Percentage efficacy} = 100 - (T \times 100/K)$$

where T = positive hairs in the test group and K = total positive hairs in the infected untreated control group. The total score for any group denotes the average count of fungus-positive hairs

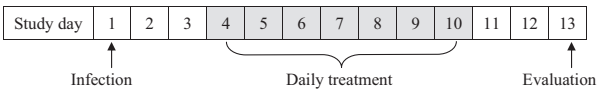

Figure 1 Experiment schedule

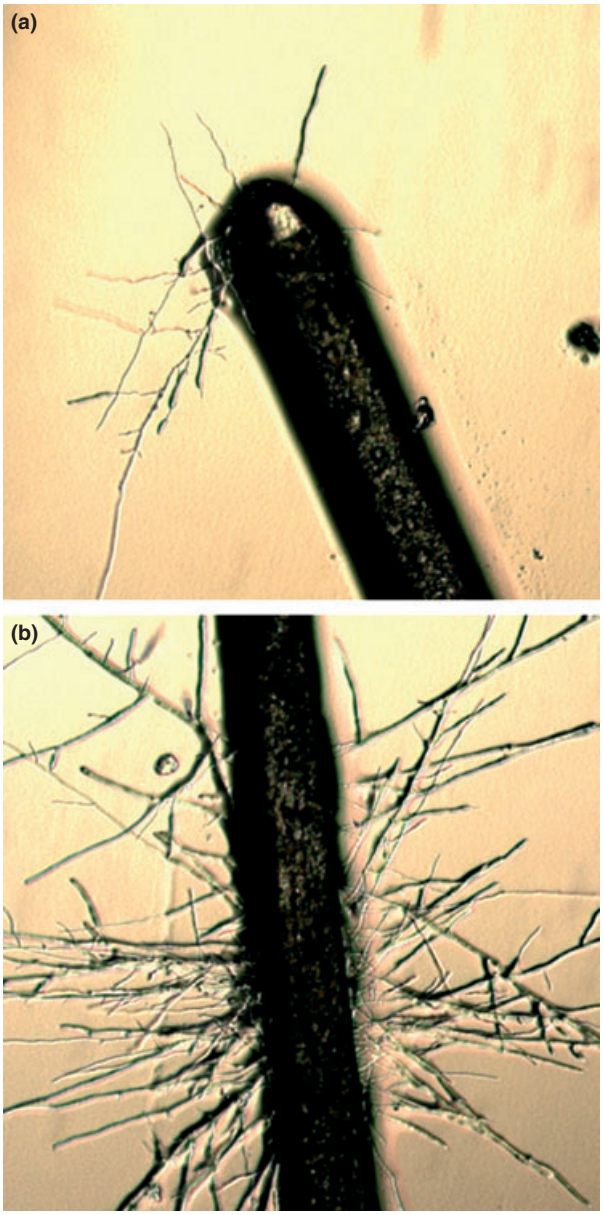

Figure 2 Guinea pig hairs implanted on potato dextrose agar showing fungal growth (a) at the hair root and (b) from the hair shaft. (Original magnification ×10)

obtained per quadrant from the animals in the same group. When hair loss prevented the collecting of samples, quadrants were recorded as having no hair and eliminated from the calculations.

Statistical analysis

Statistical analyses of data from the clinical and mycological studies were performed using SPSS, version 19.0 (Chicago, IL, USA). Significance was determined using analysis of variance (ANOVA) with a Bonferroni *post hoc* test. Outcomes were compared between the treated groups and the untreated control group to determine antifungal activity. A *P*-value of < 0.05 was considered to indicate statistical significance.

Histopathological analysis

Skin biopsy samples were obtained from one animal per treatment group, vehicle-treated group, and untreated control group on day 10 of the study. Using a disposable, sterile dermal biopsy punch (Miltex Instrument, Inc., Bethpage, NY, USA), a piece of skin, 3-mm in diameter, was obtained from an anesthetized animal representative of each group. The tissue was fixed with 10% neutral buffered formalin (Evergreen Scientific, Inc., Los Angeles, CA, USA), embedded in paraffin, and processed for histopathological examination in hematoxylin and eosin and Grocott methenamine silver (GMS) stain.

Results

Efficacy of NVC-422 in the treatment of dermatophytosis

Tables 1 and 2 show the clinical efficacy of each test compound relative to outcomes in the untreated control group. The infected areas of the untreated and vehicle control animals exhibited extensive redness, ulceration, hair loss, encrustation, and scaling. The test compound 2% NVC-422 in 1% Noveon Gel showed significant clinical efficacy compared with the untreated control ($P < 0.0001$); animals in the group treated with 2% NVC-422 in 1% Noveon Gel demonstrated the least hair loss and encrustation. This compound thus demonstrated higher clinical efficacy compared with the treatment compounds 1% NVC-422 in 1% Noveon Gel, 1.5% NVC-422 in 1% Noveon Gel, and 1% Noveon Gel ($P = 0.019$, $P = 0.050$ and $P < 0.0001$, respectively).

Outcomes of treatment with NVC-422 0.5% in 0.5% Noveon Gel, 1% NVC-422 in 1% Noveon Gel, 1.5% NVC-422 in 1% Noveon Gel, and 2% NVC-422 in 1% Noveon Gel differed significantly from outcomes in the untreated control group ($P = 0.003$, $P = 0.029$, $P = 0.012$ and $P < 0.0001$, respectively), indicating that these compounds showed significant clinical efficacy (Table 1).

Table 1 Outcomes in treatment and vehicle-treated control groups showing the clinical and mycological efficacy of treatment compounds

Test compound	Efficacy, %	Animals showing mycological cure, %
0.5% NVC-422 in 0.5% Noveon Gel	23.7[a]	86.0[a]
1% NVC-422 in 1% Noveon Gel	19.3[a]	90.5[a]
1.5% NVC-422 in 1% Noveon Gel	20.9[a]	80.5[a]
2% NVC-422 in 1% Noveon Gel	36.6[a]	97.9[a]
1% Noveon Gel	3.6	53.8[a]

[a]Comparisons with outcomes in the untreated control group were significant at $P < 0.05$.

Table 2 Outcomes in treatment and control groups showing the clinical and mycological efficacy of test compounds

Test compound	Clinical score, mean ± SD	Number of fungus-positive hairs, mean ± SD
0.5% NVC-422 in 0.5% Noveon Gel	13.60 ± 1.65	0.39 ± 0.66
1% NVC-422 in 1% Noveon Gel	14.40 ± 1.71	0.27 ± 0.60
1.5% NVC-422 in 1% Noveon Gel	14.10 ± 2.47	0.55 ± 0.99
2% NVC-422 in 1% Noveon Gel	11.30 ± 2.50	0.06 ± 0.24
1% Noveon Gel	17.20 ± 1.69	1.30 ± 1.45
Untreated control	17.80 ± 1.94	2.82 ± 1.92

SD, standard deviation.

Mycological efficacy of NVC-422

Tables 1 and 2 show the mycological outcomes of treatment with each of the test compounds compared with that in the untreated control group. The untreated control group had the highest average number of fungus-positive hairs. Our data indicate that 2% NVC-422 in 1% Noveon Gel had the highest efficacy ($P < 0.0001$) compared with the untreated control). The percentage efficacies for 0.5% NVC-422 in 0.5% Noveon Gel, 1% NVC-422 in 1% Noveon Gel, 1.5% NVC-422 in 1% Noveon Gel, 2% NVC-422 in 1% Noveon Gel and 1% Noveon Gel were 86.0%, 90.5%, 80.5%, 97.9%, and 53.8%, respectively. Although the outcome of treatment with the vehicle only compared with outcome in the untreated control group did not show the vehicle to have clinical efficacy, it slightly affected fungal growth ([mycological efficacy of 53.8% compared with the untreated control $P < 0.0001$]).

Histopathology

Fungal elements were detectable in skin sections from untreated guinea pigs infected with *T. mentagrophytes*, indicating successful infection. No fungal elements were

noted in skin sections obtained from any of the treatment groups.

Discussion

Evaluation of the efficacy of NVC-422 in the treatment of dermatophytosis using an experimental guinea pig model showed that this compound possesses potent antifungal efficacy as measured by mycological and clinical endpoints. The highest degree of clinical and mycological efficacy was demonstrated by 2% NVC-422 in 1% Noveon Gel. Previously, we used the same model to evaluate the antifungal efficacy of 8% ciclopirox applied topically.[14] This formulation is the only topical formulation approved by the US Food and Drug Administration (FDA) for the topical treatment of onychomycosis.[14] The clinical and mycological efficacy noted for 8% ciclopirox in this model is similar to those reported for the commercially available product. In this regard, our data show that 2% NVC-422 in 1% Noveon Gel demonstrated higher efficacy than 8% ciclopirox nail lacquer, both clinically and mycologically (36.6%, 97.9% versus 7.2%, 85.0%, respectively). The vehicle showed a modest mycological effect, which was consistent with prior *in vitro* susceptibility testing in which modest antifungal activity was noted (data not shown). In conclusion, our data show that NVC-422 has potent antifungal activity *in vivo*. The highest degree of clinical and mycological efficacy was shown by 2% NVC-422 in 1% Noveon Gel. Clinical evaluation of NVC-422 in the treatment of superficial infections caused by dermatophytes, including onychomycosis, is warranted.

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