Voriconazole, a safe alternative for treating infections caused by the *Chrysosporium* anamorph of *Nannizziopsis vriesii* in bearded dragons (*Pogona vitticeps*)

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Summary

Dermal and systemic infections caused by the *Chrysosporium* anamorph of *Nannizziopsis vriesii* (CANV) are highly prevalent in reptiles, resulting in severe disease and high mortality. Due to the high incidence of therapeutic failure, optimizing treatment is required. In this study, at first, the minimal inhibitory concentrations (MIC) of itraconazole, voriconazole, amphotericin B and terbinafine against thirty two CANV isolates were determined. For voriconazole, amphotericin B and terbinafine a monomodal MIC distribution was seen, whereas a bimodal MIC distribution was present for itraconazole, indicating acquired resistance in one isolate. Fourteen naturally infected bearded dragons (*Pogona vitticeps*), from the same owner, were treated orally either with itraconazole (5 mg/kg q24h) or voriconazole (10 mg/kg q24h). The clinical condition, drug plasma concentrations and the presence of CANV in skin samples were followed up. The animals were treated until complete clearance of the fungus. The plasma concentrations of voriconazole and itraconazole exceeded the minimal inhibitory concentration of the CANV isolate. Elimination of CANV was achieved in 27 and 47 days on average for itraconazole and voriconazole, respectively. Whereas only 2 out of 7 survived after itraconazole treatment, only a single animal died in the voriconazole treated group. In conclusion, based on a limited number of animals, voriconazole applied at a regimen of 10 mg/kg bodyweight (BW) q24h seems to be a safe and effective antimycotic drug to eliminate CANV infections in bearded dragons.

Keywords: *Chrysosporium* anamorph of *Nannizziopsis vriesii*, voriconazole, treatment, dermatitis, bearded dragon

Introduction

The *Chrysosporium* anamorph of *Nannizziopsis vriesii* (CANV) recently emerged as a cause of mycotic dermatitis in different reptile species such as bearded dragons (*Pogona vitticeps*)[1], chameleons (*Chameleo* spp.)[2], green iguanas (*Iguana iguana*)[3], ameiva lizard (*Ameiva chaitzami*)[4], salt-water crocodiles (*Crocodylus porosus*)[5] and snakes (*Boiga irregularis*; *Erpeton tentaculatum*)[6,7]. Very little is known about the source of this fungus, its prevalence in the environment and its virulence factors. Recent studies indicate that the CANV is not a common constituent of the mycobiota of the reptilian skin [8]. Koch's postulates have been fulfilled in veiled chameleons (*Chameleo calyptratus*)[9]. Environmental stressors such as substandard husbandry and suboptimal cage temperatures may act as predisposing factors for CANV infections in reptiles [3].

Successful treatment of reptiles infected with CANV has been achieved by administering itraconazole in a Parson's chameleon (*Chamaeleo parsonii*) and in a bearded dragon [1,2]. Ketoconazole was effective in two green iguanas [3]. However, treatment with antimycotics was often unsuccessful [1,2]. To optimize the treatment outcome, antifungal drug selection, determination and administration of the optimal dose and frequency of antimycotic drugs are essential. In general, this requires integration of knowledge regarding the drug's pharmacokinetic profile with its antimycotic properties [10].

The use of voriconazole, a novel triazole antifungal agent, has never been described for reptiles. However the *in vitro* activity of voriconazole against filamentous fungi exceeds that of itraconazole [11]. Azoles inhibit the P450-dependent 14-α-sterol demethylase by which exposed fungi become depleted of ergosterol and accumulate 14-α-methylated sterols. This leads to disruption of membrane function and structure, resulting in fungal growth inhibition [12].

The aim of this treatment study was to evaluate the *in vitro* activity of itraconazole, voriconazole, amphotericine B and terbinafine against different isolates of CANV and to compare the efficacy of itraconazole and voriconazole treatment of CANV infections in bearded dragons. In addition the plasma concentration of itraconazole and voriconazole in a group of naturally infected bearded dragons was monitored.

Material and methods

In vitro susceptibility testing

Susceptibility testing was performed based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI) document M38-A2. Thirty two isolates of CANV were tested. All of these isolates were obtained from clinically affected animals (3 green iguanas, 28 bearded dragons and 1 giant girdled lizard (*Cordylus giganteus*)).

Antifungal agents

Stock solutions of voriconazole (Pfizer Global Pharmaceuticals, Ixelles, Belgium), itraconazole (Janssen, Beerse, Belgium), amphotericin B (SPRL Bristol-Myers Squibb, Brussels, Belgium) and terbinafine hydrochlorid (Sigma-Aldrich, St Louis, USA) were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, USA), diluted to 100 times their final concentrations and stored at -70°C before use.

On the day of the test, the stock solutions were diluted 1:50 in RPMI 1640 medium buffered to pH 7.0 with morpholinepropanesulfonic acid buffer (Invitrogen, Merelbeke, Belgium), and dispensed into 96 U-shaped well microdilution trays. Each well contained 0.1 ml of the appropriate drug solution (2 x final concentration) ranging from 0.0625-32 µg/ml (final concentration 0.0313-16 µg/ml) for voriconazole, itraconazole and amphotericin B and 0.002-1 µg/ml (final concentration 0.001-0.5 µg/ml) for terbinafine.

Inoculum preparation of the Nannizziopsis vriesii isolates

The *Nannizziopsis vriesii* isolates, grown on Sabouraud dextrose agar for three weeks at 30°C, were washed with 5 ml of 1% Tween 20 in sterile phosphate buffered saline (PBS) to harvest conidia. The conidia were adjusted to a concentration of $4x10^6$ conidia/ml in PBS by hemocytometer count. These stock conidial suspensions were diluted 1:50 in RPMI 1640 medium buffered to pH 7.0 with morpholinepropanesulfonic acid buffer, and dispensed into 96 U-shaped well microdilution trays. Each well contained 0.1 ml of the diluted conidial suspensions.

Inoculum preparation of the quality control strain Candida krusei (ATCC 6258-IHEM 9560)

The *Candida* species was grown on Sabouraud dextrose agar and incubated at 35°C for 24 hours. Stock suspension was prepared in PBS, adjusted to match the turbidity of a Mc Farland 0.5 standard and further diluted 1:50 in the standard RPMI 1640 medium. The quality control strain was tested in the same manner as the *Nannizziopsis vriesii* isolates and included on the same test day.

Susceptibility testing

Each test plate contained four drug-free growth controls of standard RPMI-1640 medium without antifungal agent plus 1% DMSO and one not inoculated-drug-free control to ensure the medium's sterility. The test plates were incubated at 30°C and evaluated after 48, 72, 96 and 120 hours. Two persons read the test plates independently to minimize variation in the interpretation of MIC end points.

MIC endpoints for C. krusei

The MIC endpoint for the azoles and terbinafine was defined as the lowest concentration that produced 80% inhibition compared with the growth in the control well, but for amphotericin B, the endpoint was defined as the lowest concentration with complete inhibition of growth [13].

MIC endpoints for Nannizziopsis vriesii isolates

The MIC endpoint for the azoles and amphotericin B was defined as the lowest concentration that produced complete inhibition of growth or the first optically clear well, whereas the MIC endpoint for terbinafine was defined as the lowest concentration that produced 80% inhibition compared with the growth in the control well.

Treatment study

Animals

In September 2008 one owner presented fourteen bearded dragons with dermatitis on the head, hind limbs and ventrum at the Faculty of Veterinary Medicine, Ghent University, Belgium. Pure and abundant cultures of CANV were obtained from each animal after microbiological sampling of the lesions. On histological examination of skin samples the presence of intra-lesional hyphae was confirmed by using the Perodic Acid Shiff reaction (PAS). Polymerase chain reaction (PCR) and sequencing of the ITS1-5.8S-ITS2 region were performed for species confirmation [14]. Through search of the GenBank database, 100 % homology was found with a sequence previously published for *Nannizziopsis vriesii* AJ131681.

During the hospitalisation period the bearded dragons were housed individually. The temperature was maintained at 28°-30°C with a 12-hour photoperiod. The diet consisted of vegetables and insects and there was free access to fresh drinking water.

Treatment

The severity of the dermal lesions was evaluated for each bearded dragon based on the size and macroscopic aspect. Based on this evaluation, the fourteen bearded dragons were divided in two treatment groups so that each treatment group consisted of two animals with a distinct dermatitis, three animals with moderate lesions and two animals with a severe dermatitis. One treatment group received itraconazole (Sporanox®, Janssen-Cilag, Berchem, Belgium) orally q24h at a dosage of 5 mg/kg bodyweight (BW) and the other one voriconazole (Vfend®, Pfizer, Ixelles, Belgium) orally q24h at a dosage of 10 mg/kg BW. During the antifungal therapy, the bearded dragons were weighed once every seven days, and consecutive mycological sampling after debridement of the dermal lesions was performed.

Blood samples to determine the plasma concentrations of antifungal drugs, taken from the ventral coccygeal vein, were collected prior to the first administration of the antifungal drugs (C_{min}) and 2h after administration (estimated C_{max}) during the first three days of treatment. Thereafter blood samples were taken once weekly. Blood (1 ml) was collected into heparin coated tubes (Microvette®, Sarstedt, Nümbrecht, Germany) and centrifugated (2400*g* for 10 min at room temperature) immediately after collection. Plasma concentrations of voriconazole and itraconazole were measured using a LCMS/MS method slightly modified to the one described by Egle *et al*. (2005)[15]. The HPLC system consisted of a Thermo Surveyor MS pump Plus and a column heater module, the mass spectrometer was a TSQ-Quantum triple-quadrupole instrument equipped with an electrospray ionization source operating in the positive ion mode, both from Thermo Electron Corporation (Waltham, USA). A PLRP-S column (100Å, 5 µm, and 150x4.6 mm) from Polymer Laboratories (Varian inc., Sint-Katelijne-Waver, Belgium) was used for chromatographic separation with hydroxy-itraconazole as internal standard for the voriconazole analysis and voriconazole as internal standard for the itraconazole analysis.

A serum biochemistry profile and hematological evaluation was also performed on the blood samples after 4 days of treatment.

Necropsy

Animals which died during treatment were subjected to a necropsy. Samples from the liver, lung and skin were taken for mycological and histological examination.

Results

In vitro susceptibility testing

The results of the *in vitro* susceptibility testing of amphotericin B, itraconazole, voriconazole and terbinafine are summarized in table 1. For voriconazole, terbinafine and amphotericin B a monomodal MIC distribution was seen. However a bimodal MIC distribution was present for itraconazole, indicating acquired resistance in one isolate in the higher range of MICs.

For all isolates recovered during the antimycotic treatment in the case control study, the MIC of voriconazole was < 0.0313 µg/ml and of itraconazole < 0.0313 µg/ml.

The MICs of the quality control strain *Candida krusei* (ATCC 6258-IHEM 9560) were within the established reference MIC ranges for voriconazole, itraconazole and amphotericin B described by Barry *et al*., 2000 [13]. No reference range is available for terbinafine.

Treatment study

The effect on the mortality and the efficacy to eradicate the infection of the two treatments is presented in table 2. Five animals in the itraconazole treatment group died after 5, 20, 22, 36, 54 days of treatment, respectively. In none of these animals the fungus could be isolated from the internal organs. CANV was isolated from the skin of the lizards that died after 5 and 22 days of treatment. Neither gross nor histological lesions were observed in the liver and lungs.

For the skin samples, a chronic, multifocal, ulcerative dermatitis with intralesional hyphae was seen. One animal out of seven treated with voriconazole, died after 25 days of treatment. CANV was isolated from the liver and lungs of this animal. CANV infection was cleared in all 6 voriconazole treated animals that survived.

The mean plasma concentrations of itraconazole and voriconazole are presented in figure 1 and 2, respectively. An accumulation of itraconazole was seen in the plasma of all the animals. Prior to and following each administration of itraconazole, the established C_{min} and estimated C_{max} ranged from 527.0-7519.0 (mean = 3737.05 µg/l) and 155.9-7825.3 µg/l (mean = 3643.88 µg/l), respectively. Voriconazole plasma concentrations showed more interindividual variation than itraconazole plasma concentrations. Prior to and following each administration of voriconazole, the established C_{min} and estimated C_{max} ranged from ≥ 57.9-12704.1 (mean = 3421.8 µg/l) and ≥ 911.0-14372.1 µg/l (mean = 5738.0 µg/l), respectively.

The serum biochemical values of each bearded dragon are presented in table 3. Significantly increased levels of aspartate aminotransferase (AST) were observed in 4 animals treated with itraconazole and three animals treated with voriconazole. This is suggestive of liver damage.

Discussion

Based on a limited number of animals, this study suggests voriconazole 10 mg/kg BW q24h to be a safe and effective treatment for CANV infections in bearded dragons. Despite the long duration to clear the fungal infection, only one animal, suffering a systemic infection, died during treatment with voriconazole. Since it is not possible to predict how long the therapy should be administered, a weekly mycological follow-up using a swab from the lesions is recommended.

In this study the plasma concentrations of voriconazole were followed up during 18 days. Until now no pharmacokinetic studies of voriconazole in reptiles have been described in the literature. The results show that, as in humans and birds [17,18], the interindividual variability of voriconazole plasma concentrations in bearded dragons is high. During the therapy, an increased elimination of voriconazole was seen. This may be due to the induction of liver enzymes. This induction is also seen in pigeons, mice, rats, dogs, and African grey parrots [12,19,20,21]. In rats and dogs a dose-related increase in hepatic cytochrome P450 content in livers and an associated increase in relative liver weight was consistent with auto induction of voriconazole metabolization, thereby leading to a significant increase in clearance [20].

Itraconazole was very adequate in eliminating the CANV infection. After an average of four weeks, the fungus could not be re-isolated from the lesions. However, only two animals out of seven survived the therapy. Based on the blood biochemistry, toxicity due to itraconazole administration was suggested. This however could not be demonstrated via histological examination of the liver and lungs. In humans a concentration of 17.1 mg/l itraconazole in blood plasma results in a high probability of toxicity [22]. Bowman *et al*. (2007)[1] reported mortality in bearded dragons after itraconazole administration, but, similar to this case, toxicity could not be proven. This author suggested pulse therapy for itraconazole in reptiles. However, Edgerton and Griffin (2009)[23] described a non-successful treatment of a CANV infection in a bearded dragon with pulse therapy of itraconazole. Treatment regimens with a lower dosage or a longer dosing interval with a potential reduction of the risk of itraconazole toxicity should be further investigated.

MIC values in *Chrysosporium* species for itraconazole have been reported by de Hoog *et al*. (2000)[24] and ranged from 0.12 µg/ml to 0.63 µg/ml. Paré *et al*. (2005)[25] reported low but not further specified MICs for amphotericin B, terbinafin, itra- and voriconazole in CANV

isolates. In our study one isolate out of thirty two showed acquired resistance to itraconazole. A large scale study should be performed in order to get a better knowledge of the percentage of resistant CANV strains, their resistance mechanisms and the origin of resistance.

In conclusion, based on a limited number of animals, despite interindividual variable pharmacokinetic properties and possible induction of liver enzymes voriconazole seems to be a safe and effective antimycotic drug to treat CANV infections in bearded dragons at a dose of 10 mg/kg BW q24h.

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346 Table 1: *In vitro* activity of selected antifungal agents against 32 isolates of the

347 *Chrysosporium* anamorph of *Nannizziopsis vriesii* from captive lizards.

Antifungal agent	Range	MIC (µg/ml) 50%	90%
Amphotericin B	0.0313-16	2	2
Itraconazole	0.0313-16	0.0313	0.25
Voriconazole	0.0313-16	0.0313	0.0625
Terbinafine	0.001-0.5	1	2

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349 MIC=Minimal Inhibitory Concentration
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Table 2: Overview of the treatment duration, clearance of the infection and mortality in bearded dragons naturally infected with CANV after treatment with itraconazole or voriconazole.

	Treatment duration (days)		**Clearance of**	**Outcome of**
	Range	**Average**	**CANV infection**	**treatment**
Itraconazole (5 mg/kg BW q 24h)	14-42	27	5/7	2/7 survived
Voriconazole (10 mg/kg BW q24h)	28-64	47	6/7	6/7 survived

BW=BodyWeight

373 Table 3: Serum biochemical profile of the bearded dragons during the treatment.

Treatment		**Itraconazole**		**Voriconazole**	
	Reference value[a]	Average	(Range)	Average	(Range)
AST (IU/l)[b]	27± 23	192,7	(15 – 431)	157,4	(24 – 446)
UA (mg/dl)[b]	4,4 ± 2,6	2,4	(0,9 - 3,5)	2,8	(0,9 - 5,2)
Glu (mg/dl)[b]	201 ± 52	186,2	(148 – 251)	228,8	(191 – 282)
Ca (mg/dl)[b]	16,2 ± 11,2	8,6	(5,1 – 14)	9,5	(7,9 - 10,9)
Phos (mg/dl)[b]	5,6 ± 2,5	6,5	(5,9 - 7,6)	7,9	(5,2 - 10,1)
TP (g/dl)[b]	5,0 ± 1,4	4,4	(3,7 - 5,3)	5,2	(4,4 - 6,7)
Alb (g/dl)[b]	2,6 ± 0,8	1,86	(1,5 - 2,4)	2,3	(1,5 - 2,7)
Glob (g/dl)[b]	2,3 ± 0,9	2,57	(2,2 - 3,2)	2,96	(2,4 – 4)
K^+ (mEq/l)[b]	3,8 ± 1,2	5,6	(3,4 - 7,4)	4,8	(1 - 9,2)
Na^+ (mEq/l)[b]	156 ± 11	168,4	(164 – 172)	170,7	(167 – 180)

374 [a]Reference value according to Pollock *et al*.[16].

375 [b]AST: aspartate aminotransferase, UA: uric acid, GLU: glucose, Ca: calcium, Phos:
376 phosphorus, TP: total protein, ALB: albumin, GLOB: globulin, K+: potassium, Na+: sodiu

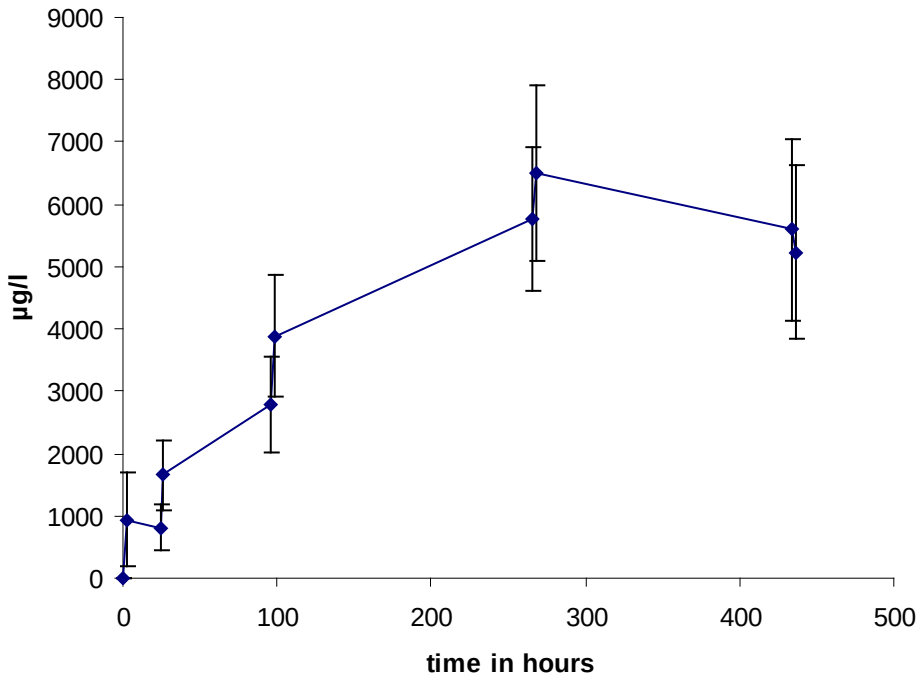
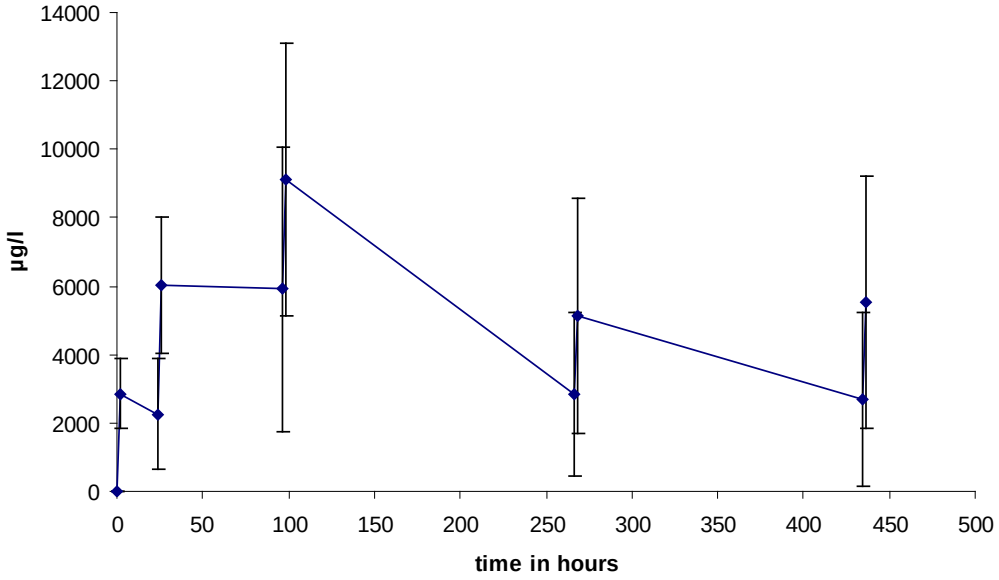


Figure 1: Average (± SD) plasma concentration of itraconazole (µg/l) in bearded dragons (n=6) after oral administration of itraconazole at a dose of 5 mg/kg SID.



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390 Figure 2: Average (± SD) plasma concentration (µg/l) of voriconazole in bearded dragons

391 (n=7) after oral administration of voriconazole at a dose of 10 mg/kg SID

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