VT-1598 inhibits the *in vitro* growth of mucosal *Candida* strains and protects against fluconazole-susceptible and -resistant oral candidiasis in IL-17 signalling-deficient mice

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Background: Chronic mucocutaneous candidiasis (CMC) treatment often induces drug resistance, posing long-term challenges. A novel broad-spectrum fungal CYP51 inhibitor, VT-1598, specifically targets fungal CYP51, but not human CYP enzymes.

Objectives: To determine the efficacy of VT-1598 in the treatment of oral *Candida* infection caused by fluconazole-susceptible and -resistant clinical isolates.

Methods: The MICs of VT-1598 and fluconazole for 28 *Candida* isolates recovered from patients with inherited CMC were determined using CLSI M27-A3 and M27-S4 guidelines. Plasma and tongue VT-1598 or fluconazole concentrations were measured in mice following oral administration to determine tissue distribution. Tongue fungal load was determined in IL-17 signalling-deficient *Act1*^{-/-} mice following sublingual *Candida albicans* infection and oral treatment with fluconazole or VT-1598.

Results: Among the 28 *Candida* isolates, 10 (36%) had fluconazole MICs of ≥ 4 mg/L, whereas VT-1598 demonstrated potent *in vitro* activity against all isolates (MIC₉₀, 0.125 mg/L). After oral administration, VT-1598 levels in mouse plasma and tongue were significantly greater than those of fluconazole. *In vivo*, VT-1598 exhibited significant efficacy against fluconazole-susceptible and -resistant *C. albicans*, even at low drug doses. Furthermore, after a 10 day washout period, tongue fungal burdens in fluconazole-treated mice returned to vehicle control levels, whereas, in contrast, they were undetectable in mice treated with VT-1598.

Conclusions: VT-1598 effectively controls *in vitro* growth of mucosally derived *Candida* clinical isolates, including fluconazole-resistant strains. *In vivo*, VT-1598 eliminates *C. albicans*, even after a long washout period or at low doses. Therefore, VT-1598 is a promising drug candidate that may significantly improve treatment options for CMC patients.

Introduction

Antifungal drug resistance is commonly acquired in patients with chronic mucocutaneous candidiasis (CMC), including patients with primary immunodeficiency diseases (PIDDs) such as autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) and those with AIDS or recurrent vulvovaginal candidiasis.^{1–4} Antifungal resistance mostly occurs against azole drugs, the most common therapy for chronic *Candida* infections. In patients with

azole resistance, echinocandins or amphotericin B may be used,⁵ but these are parenteral medications and their chronic use is also associated with emergence of resistance or with renal toxicity, respectively. A new class of antifungal drugs has been developed that targets fungal 14 α -demethylase cytochrome P450 (CYP51), the same target as azole drugs, but does not cross-react with the human CYP enzymes,⁶ a known limitation with azoles. We have previously shown a role for VT-1161 in the protection of mice against oropharyngeal candidiasis (OPC),⁷ and this drug has

completed Phase II clinical trials for the treatment of recurrent vulvovaginal candidiasis (NCT02267382). VT-1598 is another highly selective fungal CYP51 inhibitor⁸ that has been granted Fast Track designation by the FDA for the treatment of coccidioidomycosis and has *in vitro* activity against a variety of fungal species, including azole-resistant strains.⁹ However, whether VT-1598 protects against OPC has not been assessed. Herein, we aimed to determine the ability of VT-1598 to inhibit the *in vitro* growth and confer *in vivo* protection against OPC caused by fluconazole-susceptible and -resistant *Candida* isolates.

Materials and methods

In vitro susceptibility testing

Twenty-eight *Candida* mucosal isolates obtained from APECED patients due to autoimmune regulator (AIRE) deficiency¹ were identified via MALDI-TOF MS and tested for susceptibility to fluconazole and VT-1598. The MIC was the concentration that inhibited growth at 50% compared with the 'no drug' control after a 24 h incubation. MIC values were determined based on CLSI microdilution guidelines (M27-A3 and M27-S4)^{10,11} once per strain and compared with the quality control strains SC5314 and ATCC 90028, as previously described.⁷ The MICs at which 50% (MIC₅₀) or 90% (MIC₉₀) of the isolates were inhibited were calculated.

Mouse model

Age- and sex-matched *Act1*^{-/-} (also known as *Traf3ip2*^{-/-} or *Ciks*^{-/-}) mice, which lack IL-17 receptor signalling¹² and are susceptible to OPC,¹³ were used for all experiments. OPC was induced using the clinical isolates Y72 (fluconazole susceptible) or Y37 (fluconazole resistant) and tongue fungal load was determined as previously described.^{7,14,15} In all experiments, the lower limit of cfu detection was 0, as the entire tongue homogenate was plated. All experiments were completed according to guidelines set forth by the Guide for the Care and Use of Laboratory Animals under a protocol approved by the NIAID Animal Care and Use Committee.

Fungal DNA analysis

In order to determine whether there are mutations in the Y37 *Candida albicans* isolate that are associated with fluconazole resistance, sequencing of the fungal DNA was conducted. Genomic DNA was isolated from overnight cultures using a DNA fungal miniprep kit following supplier instructions (Zymo Research, Irvine, CA, USA). DNA was amplified with primers targeting *ERG11*, *TAC1*, *MRR1* and *UPC2*, as listed in Table S1 (available as Supplementary data at JAC Online). PCR amplicons were purified using a sequencing clean-up kit (Zymo Research) and sequenced (Macrogen Corp., Brooklyn, NY, USA) with gene-specific primers (Table S1). Sequencing results were analysed by SeqMan Pro 14 (Lasergene by DNASTAR, Madison, WI, USA) and compared with the *C. albicans* SC5314 sequenced strain (<http://candidagenome.org/>).

Antifungal drug administration

Fluconazole (25, 8 or 3.2 mg/kg, LKT Laboratories, Inc.) or VT-1598 (20, 8 or 3.2 mg/kg, Viamet Pharmaceuticals, Inc.) was resuspended in 1% carboxymethylcellulose and administered via oral gavage starting 18 h post-infection and continued every 24 h thereafter at days 2, 3 and 4 post-infection. VT-1598 was supplied as the tosylate salt, with the correction factor of 1.3 used to determine doses; all doses listed are that of the free base. As a vehicle control, mice were given 1% carboxymethylcellulose in the same treatment scheme. Animals were sacrificed and tongues were collected and prepared for drug level determination or cfu assessment at day 5 or 14 post-infection, depending on the experimental design. Drug

levels were determined in tongue homogenates and plasma using LC-MS/MS, as previously described.¹⁶

Statistical analysis

Mann-Whitney *U*-tests or unpaired *t*-tests were used, as appropriate, to determine statistical significance using GraphPad Prism 7 software. Data are presented as the mean ± SEM. A *P* value of ≤0.05 was considered significant.

Results

VT-1598 is highly effective in inhibiting the *in vitro* growth of clinical *Candida* isolates

In order to determine whether VT-1598 and fluconazole inhibit *Candida* growth *in vitro*, the MIC₅₀ and MIC₉₀ values were determined for 28 *Candida* isolates that were obtained from mucosal sites of APECED patients. These isolates included 22 *C. albicans*, 3 *Candida glabrata* and 1 each of *Candida utilis*, *Candida dubliniensis* and *Candida krusei*. Importantly, 10 out of 28 (36%) of the isolates had fluconazole MICs of ≥4 mg/L (Table 1). These isolates included 6 out of 22 (27%) *C. albicans*, all 3 *C. glabrata* (100%) and the *C. utilis* and *C. krusei* isolates. In contrast, VT-1598 demonstrated potent *in vitro* activity against all 28 isolates (MIC range = 0.03125–0.125 mg/L). The MIC₅₀ and MIC₉₀ values of VT-1598 were 0.0625 and 0.125 mg/L, respectively, whereas the MIC₅₀ and MIC₉₀ values of fluconazole were 0.5 and 32 mg/L, respectively. Furthermore, consistent with a CYP51 inhibitor,¹⁷ VT-1598 was fungistatic *in vitro* against the *C. albicans* ATCC 90028 strain in a time-kill assay (data not shown). These data indicate that VT-1598 effectively controls the *in vitro* growth of *Candida* isolates from patients with CMC, including strains with high MICs of fluconazole.

VT-1598 is present to a greater extent than fluconazole in the plasma and tongue after oral administration in *Act1*-deficient mice infected with *C. albicans*

The potent *in vitro* anti-*Candida* activity of VT-1598 prompted us to further examine the efficacy of the drug *in vivo* during OPC. Hence, we first assessed the levels of VT-1598 in plasma and tongue (the site of infection in the OPC model) relative to fluconazole after oral administration. We used *Act1*^{-/-} mice, which are unable to signal through the IL-17RA/IL-17RC receptors and therefore are a good model of mucosal *Candida* susceptibility seen in patients with PIDDs that are deficient in the IL-17 pathway.^{18,19} *Act1*^{-/-} mice were infected with the *C. albicans* isolate Y72, which has been shown to infect WT and *Act1*^{-/-} mice,^{7,14,15} and VT-1598 (20 mg/kg) or fluconazole (25 mg/kg) was administered via oral gavage starting the following day and once daily thereafter for a total of four doses. One day after the last dose (i.e. day 5 post-infection), the concentration of VT-1598 or fluconazole was determined in the plasma and tongue; these levels would therefore reflect the *C*_{min} values under the once-daily dosing regimen. Plasma levels of VT-1598 were ~32-fold greater than fluconazole (10.1±1.0 versus 0.32±0.039 mg/L, respectively, *P* < 0.0001; Figure 1a). Furthermore, VT-1598 levels in the tongue were ~78-fold greater than that of fluconazole (24.3±4.4 versus 0.31±0.031 µg/g, respectively, *P* < 0.05; Figure 1b). These data show that oral doses of VT-1598 result in higher concentrations than fluconazole in the

Table 1. MIC values of VT-1598 and fluconazole for *Candida* clinical isolates

Isolate identifier	Species	MIC (mg/L)	
		VT-1598	fluconazole
Y31	<i>C. albicans</i>	0.125	32
Y37	<i>C. albicans</i>	0.125	64
Y42	<i>C. albicans</i>	0.0625	0.125
Y43	<i>C. albicans</i>	0.03125	0.25
Y46	<i>C. glabrata</i>	0.03125	8
Y47	<i>C. albicans</i>	0.03125	0.25
Y48	<i>C. albicans</i>	0.0625	0.5
Y49	<i>C. glabrata</i>	0.0625	128
Y51	<i>C. albicans</i>	0.125	4
Y54	<i>C. glabrata</i>	0.0625	128
Y55	<i>C. albicans</i>	0.125	16
Y57	<i>C. albicans</i>	0.03125	4
Y72	<i>C. albicans</i>	0.0625	0.5
Y75	<i>C. albicans</i>	0.0625	1
Y79	<i>C. albicans</i>	0.03125	0.5
Y82	<i>C. utilis</i>	0.125	4
Y83	<i>C. albicans</i>	0.125	2
Y84	<i>C. albicans</i>	0.0625	0.5
Y88	<i>C. albicans</i>	0.0625	0.5
Y92	<i>C. dubliniensis</i>	0.0625	0.25
Y93	<i>C. albicans</i>	0.0625	0.5
Y107	<i>C. albicans</i>	0.0625	0.5
Y111	<i>C. albicans</i>	0.0625	0.5
Y125	<i>C. krusei</i>	0.125	16
Y152	<i>C. albicans</i>	0.0625	0.125
Y153	<i>C. albicans</i>	0.125	0.125
Y160	<i>C. albicans</i>	0.125	1
Y162	<i>C. albicans</i>	0.0625	1

A total of 28 *Candida* isolates were tested and the MIC values were determined based on CLSI M27-A3 and M27-S4.

plasma and tongues of *Candida*-infected mice, at least as measured by the C_{min} value.

VT-1598 provides effective and long-lasting protection against OPC in vivo

Given the potent *in vitro* activity and high oral tissue and plasma levels of VT-1598, we then assessed the ability of this drug to provide protection in an *in vivo* model of OPC using several approaches. We first tested the capacity of VT-1598 and fluconazole to protect against the fluconazole-susceptible strain Y72 (MIC of VT-1598, 0.0625 mg/L; MIC of fluconazole, 0.5 mg/L; Table 1). Both fluconazole and VT-1598 provided significant protection against this strain; however, VT-1598 completely cleared *C. albicans*, while fluconazole treatment led to low, but measurable, *C. albicans* in the tongue (Figure 1c). Next, we determined the ability of VT-1598 to protect against a fluconazole-resistant strain, Y37 (MIC of VT-1598, 0.125 mg/L; MIC of fluconazole, 64 mg/L; Table 1). Not surprisingly, fluconazole was unable to provide protection against this strain.

Instead, VT-1598 completely cleared *C. albicans* from the tongue of 6 out of 7 animals (Figure 1d).

In order to gain insight into azole resistance mechanisms within the fluconazole-resistant Y37 isolate, which VT-1598 is able to overcome, we sequenced *ERG11*, *TAC1*, *MRR1* and *UPC2* to identify potential mutations. Y37 was found to have an Erg11 amino acid substitution (G464S) that has been proven to confer fluconazole resistance.^{20,21} Interestingly, Y37 also contained a duplication event (S171delinsQSNPP) in *MRR1*. Tandem repeats of amino acids NPQS at the MDR regulator Mrr1 locus have been reported in fluconazole-resistant *C. albicans* isolates.²² Three mutations were observed in *TAC1* (L131I/L, F189S, I558V/I), although the significance of these mutations in promoting fluconazole resistance is unknown. In contrast, no *UPC2* mutations were identified. Therefore, the identified *ERG11* and *MRR1* genetic variants likely account for the elevated fluconazole MIC for Y37 and the lack of *in vivo* efficacy of fluconazole during OPC caused by this strain. These data suggest that VT-1598 is able to overcome at least two reported fluconazole resistance mechanisms and are consistent with an extensive analysis of several fluconazole-resistant, VT-1598-susceptible *Candida* isolates performed by P. D. Rogers, N. P. Wiederhold and E. P. Garvey (unpublished data).

We next asked whether lower doses of VT-1598 (i.e. 3.2 or 8 mg/kg) still provided effective control of the mucosal infection. Impressively, administration of either drug dose in *Act1*^{-/-} mice led to elimination of the infection, with only 1 out of 6 evaluated mice having low-level detectable cfu at the 3.2 mg/kg dose (Figure 1e); in contrast, administration of 3.2 or 8 mg/kg of fluconazole resulted in dose-dependent, albeit lesser, protection compared with the corresponding doses of VT-1598 (Figure 1e). Lastly, because patients with PIDs that manifest with CMC require long-term antifungal therapy and frequently relapse soon after discontinuation of azole treatment, the ability of VT-1598 to offer long-term protection against OPC was examined. *Act1*^{-/-} mice were infected with Y72 and treated with 25 mg/kg fluconazole or 20 mg/kg VT-1598 for 4 days as above; then, after the last dose, mice were left untreated for 10 days and cfu were determined at day 14 post-infection to examine whether microbiological relapse would be apparent. Mice given vehicle or fluconazole had extremely high cfu in the tongue with a slight, but statistically significant, decrease in the fluconazole-treated animals as compared with vehicle-treated mice. In sharp contrast, mice given VT-1598 did not have measurable cfu in the tongue after the 10 day washout period (Figure 1f). We measured drug levels in tongue and plasma 10 days after the last dose of VT-1598 at day 14 post-infection. We found that 5 of the 6 tongue samples showed VT-1598 drug levels below the lower limit of quantification (0.0029 µg/g), with only one sample measuring detectable VT-1598 at 0.0039 µg/g. Seven of the eight plasma samples had detectable, but very low, levels of VT-1598 (0.004±0.002 mg/L), with one sample showing a higher level (0.04 mg/L). Thus, all VT-1598 levels were below the MIC for this isolate (0.0625 mg/L), with all but one being ≥10-fold below the MIC. Therefore, although VT-1598 is not fungicidal *in vitro*, these data suggest that VT-1598 may possess a post-antifungal effect (PAE) *in vivo*. Regardless, when taken together, these data indicate that VT-1598 confers significant *in vivo* protection against fluconazole-susceptible and -resistant *C. albicans* strains. Furthermore, low doses of the drug are able to clear *C. albicans*

Figure 1. VT-1598 provides remarkable protection against oral candidiasis in IL-17 signalling-deficient mice. (a–d) *Act1*^{−/−} mice were infected with *C. albicans* and treated with vehicle, fluconazole (25 mg/kg) or VT-1598 (20 mg/kg) starting at 18 h post-infection and continued every 24 h thereafter at days 2, 3 and 4 days post-infection. At day 5 post-infection, 24 h after the last drug dose, plasma (a) and tongue (b) drug concentrations were determined after infection with the fluconazole-susceptible *C. albicans* isolate Y72. Tongue cfu were determined in *Act1*^{−/−} mice infected with Y72 (c) or the fluconazole-resistant *C. albicans* isolate Y37 (d) at day 5 post-infection. (e) Tongue cfu were determined at day 5 post-infection from *Act1*^{−/−} mice infected with the fluconazole-susceptible *C. albicans* isolate Y72 and given VT-1598 or fluconazole starting at 18 h post-infection and continued every 24 h thereafter at days 2, 3 and 4 days post-infection, but with 3.2 or 8 mg/kg dosing. (f) *Act1*^{−/−} mice were infected with Y72 and treated with vehicle, fluconazole (25 mg/kg) or VT-1598 (20 mg/kg) starting at 18 h post-infection and continued every 24 h thereafter at days 2, 3 and 4 post-infection. Ten days after the last dose (day 14 post-infection), tongue fungal load was determined. Data were combined from 2–4 independent experiments with a total of 4–13 mice per group. Data were analysed using Mann–Whitney *U*-tests or unpaired *t*-tests, where appropriate. *, **, *** and **** indicate that the groups differ at *P* < 0.05, < 0.01, < 0.001 and < 0.0001, respectively.

from the tongue and VT-1598 provides long-lasting protection against OPC relapse even after drug discontinuation.

Discussion

In this study, we demonstrate the promising preclinical efficacy of the tetrazole VT-1598 against mucosal candidiasis in the setting of

IL-17 deficiency. We found that VT-1598 is potent in its ability to inhibit the *in vitro* growth of mucosally derived fluconazole-susceptible and -resistant *Candida* isolates. After oral administration, VT-1598 levels were significantly greater than fluconazole levels in the plasma and tongues of mice with OPC. This enhanced *in vitro* efficacy and tissue exposure led to profound protection against fluconazole-susceptible and -resistant *C. albicans* isolates

during OPC, even at low drug doses. Of note, a preliminary investigation of fluconazole resistance mechanisms that VT-1598 may be immune to suggest at least some mutations in the *ERG11* and *MRR1* genes. Importantly, VT-1598 offered long-lasting protection from relapse after drug discontinuation in this OPC model.

Our data indicate that, as with another tetrazole-based fungal CYP51 inhibitor (VT-1161),^{7,23} VT-1598 has potent antifungal activity against azole-susceptible and -resistant *Candida* strains. In addition, the long-lasting protection against *Candida* infection relapse following drug discontinuation suggests an *in vivo* PAE and is a very appealing feature, given that patients with CMC develop rapid relapses upon azole discontinuation. Future pre-clinical studies should probe how long the PAE may last and if true eradication of yeast from tongue tissue has occurred. However, for the ultimate validation, clinical studies would be required to ascertain the medical relevance of this *in vivo* finding. As with all studies, the present study has limitations. We only assessed the efficacy of VT-1598 during oral candidiasis; future studies will be required to determine whether VT-1598 also protects against systemic candidiasis or other mucosal *Candida* infections seen in humans, such as vaginal or cutaneous disease. VT-1598 is in the process of entering Phase I human clinical trials. These and other future human studies will be required to test the safety and efficacy of this new tetrazole for use in patients with mucosal *Candida* infections.

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Transparency declarations

C. M. Y., R. J. S. and E. P. G. are employees and O. J. C. was an employee of the pharmaceutical company Viamet Pharmaceuticals, Inc., which is the producer of VT-1598, and each has an ownership interest in Viamet Pharmaceuticals Holdings, LLC, the parent company of Viamet Pharmaceuticals, Inc. M. S. L. received funding support from Viamet Pharmaceuticals, Inc. for these studies. All other authors: none to declare.

Supplementary data

Table S1 is available as [Supplementary data](#) at JAC Online.

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