Efficacy of the Clinical Agent VT-1161 against Fluconazole-Sensitive and -Resistant Candida albicans in a Murine Model of Vaginal Candidiasis

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Running Title: VT-1161 Efficacy in Murine Vaginal Candidiasis

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Abstract

Vulvovaginal candidiasis (VVC) and recurrent VVC (RVVC) remain major health problems for women. VT-1161, a novel fungal CYP51 inhibitor which has potent antifungal activity against fluconazole-sensitive *Candida albicans*, retained its *in vitro* potency (MIC\(_{50} \leq 0.015\) and MIC\(_{90} = 0.12\) μg/ml) against 10 clinical isolates from VVC or RVVC patients resistant to fluconazole (MIC\(_{50} = 8\) and MIC\(_{90} = 64\) μg/ml). VT-1161 pharmacokinetics in mice displayed a high volume of distribution (1.4 L/kg), high oral absorption (73%) and long half-life (>48 h), and showed rapid penetration into vaginal tissue. In a murine model of vaginal candidiasis using fluconazole-sensitive yeast, oral doses as low as 4 mg/kg VT-1161 significantly reduced fungal burden 1 and 4 days post-treatment (P values <0.0001). Similar VT-1161 efficacy was measured when an isolate highly resistant to fluconazole (MIC = 64 μg/ml) but fully sensitive *in vitro* to VT-1161 was used. When an isolate partially sensitive to VT-1161 (MIC = 0.12 μg/ml) and moderately resistant to fluconazole (MIC = 8 μg/ml) was used, VT-1161 remained efficacious, whereas fluconazole was efficacious on day 1 but did not sustain efficacy 4-days post-treatment. Both agents were inactive in treating an infection with an isolate that demonstrated weaker potency (2 and 64 μg/ml for VT-1161 and fluconazole, respectively). Finally, plasma concentrations of free VT-1161 were predictive of efficacy when in excess of in vitro MIC values. These data support the clinical development of VT-1161 as a potentially more efficacious treatment of VVC and RVVC.
Introduction

Vulvovaginal candidiasis (VVC) is a common mucosal fungal infection in women of childbearing age (1). Epidemiology studies where both yeast cultures and symptoms were confirmed (2,3) suggest a prevalence of several million infections in the U.S annually. This prediction is consistent with the estimate of 10 million annual physician visits due to vaginal symptoms and the approximate percentage of about a third of such infections being caused by yeast (4). Many of these visits represent multiple vaginal yeast infections in any individual in a given year, with four or more infections per year formally defined as recurrent VVC (RVVC). RVVC is regarded as a chronic condition with a serious impact on quality of life (QOL) (5). A recent survey determined its QOL index score being equal to that for asthma or COPD and worse than for headache/migraine (6).

Pharmaceutical treatments of fungal infections are classified by mechanism of action of a given drug (7). For most mucosal yeast infections such as VVC, the class of choice are theazole antifungal drugs (e.g., fluconazole, itraconazole, clotrimazole, etc.). These drugs target fungal CYP51 which is required for the biosynthesis of lanosterol, a key component of the fungal cell membrane (8). For VVC treatment,azole antifungals are administered both topically and systemically, with both routes largely successful in treating up to 90% of uncomplicated disease (1). A retrospective review showed no efficacy difference between routes but a preference for oral treatment (9).

Treatment of RVVC is less successful. The commonly prescribed oral fluconazole as a maintenance regimen (5) was shown in a randomized longitudinal clinical study to achieve 91% clinical efficacy of maintaining clinical remission during 6-
month prophylaxis, but only to 42% efficacy 6 months after the final dose (10). These data suggest that maintenance fluconazole is largely suppressive in nature, i.e., when therapy is stopped, symptomatic infection returns, and are consistent with relapse being most often due to the same *Candida* strain (10, 11).

In addition to the difficulty in treating recurrent disease, there are indications that drug resistance is increasing, particularly with regard to *C. albicans*, but also reflects increases of percentages of *Candida* spp. that are intrinsically less susceptible to antifungal drugs (12). *Candida albicans* is the predominant yeast species isolated from VVC patients (1) and is inherently sensitive to fluconazole. However, the percentage of non-*albicans Candida* spp. that are inherently less sensitive to fluconazole (e.g., *C. glabrata*) may be increasing (13, 14). Additionally, a higher percentage of non-*albicans* *Candida* spp (42%) was measured in RVVC patients than in patients with sporadic infrequent VVC (20%) (15), possibly leading to greater difficulty in treating RVVC due to a higher percentage of species with less susceptibility to azole antifungals.

We have recently described a novel fungal CYP51 inhibitor VT-1161 (16) that was designed for greater selectivity relative to off-target human CYP enzymes while retaining the same or greater potency for the fungal CYP51 target (17). The potency of VT-1161 against *C. albicans* CYP51 in a cellular assay was ≤0.5 nM compared to *in vitro* IC50 values of ~100 μM or greater against human CYP51 and key xenobiotic-metabolizing CYPs present in human liver microsomes (e.g., CYP2C9, 2C19, and 3A4), and its *in vitro* MIC value against wild-type fluconazole-sensitive *C. albicans* was 0.002 μg/ml (17). We present here the *in vitro* MIC potency of VT-1161 against several clinical isolates that have reduced susceptibility or were fully resistant to fluconazole.
and also the *in vivo* activity of VT-1161 in a murine model of vaginal candidiasis using fluconazole-sensitive wild-type or select fluconazole-resistant *C. albicans* isolates.
Materials and Methods

For the murine vaginal candidiasis model, female CBA/J mice, 8-10 weeks old and weighing ~20 g were obtained from the National Institutes of Health (NCI, Frederick, MD). All animals were housed and handled according to institutionally recommended guidelines. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the LSU Health Sciences Center.

*Candida albicans* ATCC 3153A strain was obtained from the ATCC (Rockville, MD). All other *C. albicans* isolates with varying susceptibilities to fluconazole were obtained from the Wayne State Vaginitis Clinic microbiology laboratory organism bank; these isolates were from patients with VVC or RVVC. Cultures were maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, MI) plates at 4 °C and grown to stationary phase in phytone-peptone broth (overnight culture at 25 °C). VT-1161 was supplied by Viamet Pharmaceuticals, Inc (Durham, NC); estradiol valerate, fluconazole, and Cremaphor EL were purchased from Sigma-Aldrich (St. Louis, MO). Single-use rapid-equilibrium dialysis (RED) plates were from Thermo Fisher Scientific (Walthan, MA).

Mouse plasma was from Biochemed (Winchester, VA) and microsomes were from Life Technologies (Grand Island, NY).

**In vitro susceptibility test.** Antifungal susceptibility tests were performed using a broth microdilution method, according to CLSI document M27-A3 (2008). Range of fluconazole was from 0.125 to 64 μg/ml, and range of VT-1161 was from 0.015 to 8 μg/ml. A 0.1-ml yeast inoculum of 1.5 (± 1.0) X 10³ cells/ml in RPMI 1640 medium was added to each microdilution well. The trays were then incubated at 35 °C for 48 h. The MICs were read as the lowest antifungal concentration for both VT-1161 and
fluconazole with substantially lower turbidity (~80% growth reduction) compared to
growth in the antifungal-free growth well. MICs were determined in duplicate, with
either no difference in MICs or at most 1-dilution different; if 1-dilution different, than the
lower value was reported.

**Pharmacokinetic (PK) studies.** Single-dose oral and i.v. PK of VT-1161 was
determined in mice at TCG Lifesciences (Kolkata, India). A dose of 5 mg/kg VT-1161 in
20% Cremophor EL was administered by oral gavage and a dose of 2 mg/kg VT-1161
in 20% Cremophor EL was administered to Balb/c female mice (age 6-8 wk, weight 19-
21 g) (N=3/time point), and plasma concentrations (for oral and i.v. doses) and vaginal
tissue concentrations (for oral dose) were determined at 0.5, 1, 2, 4, 8, 24, and 48 h.
Pharmacokinetic parameters were determined for individual animals from plasma
concentration-time data using non-compartmental modeling (NCA Model 201 for
intravenous administration or NCA Model 200) in WinNonlin Professional
(PharsightCorp., Mountain View, California), version 5.3. Plasma protein binding was
determined at 2.5 μg/ml VT-1161 in triplicate using RED plates for binding dialysis and
LC/MS-MS for quantification. Microsomal stability was determined in incubations of 1
μM VT-1161 in 1 mg/ml mouse liver microsomes at 37 °C at 5, 20, 35, and 65 minutes,
using LC/MS-MS for quantification and % remaining referenced to the zero time point.

**Murine vaginitis model.** The estrogen-dependent model has been described
previously (18). Briefly, animals were injected subcutaneously with estradiol valerate
(0.1 mg dissolved in 100 μl sesame oil) 3 days prior to and 4 days after vaginal
inoculation. One day prior to inoculation, a blastospore culture was prepared of the C.
*albicans* isolate(s) to be used in the study. On the day of inoculation, blastospores were
collected and washed once with phosphate-buffered saline (PBS) and resuspended at 
2.5 × 10^6/ml in PBS for an inoculum of 5 × 10^4 cell/20 μl PBS. For inoculation, animals 
were anesthetized "to effect" by isoflurane inhalation. To anesthetized animals, 5 × 10^4 
blastospores in 20 ml PBS was introduced into the vagina, using a pipetman. Oral drug 
treatments via once-daily gavage of VT-1161, fluconazole, or vehicle (20% Cremaphor 
EL) began on day 3 post-inoculation and continued through day 6 (i.e., four days of 
treatment). There were 10 animals in each dose group. On day 7, animals were 
anesthetized and the vaginal cavity lavaged with 100 μl of PBS. The lavage fluid was 
examined microscopically for yeast and cultured for enumeration of organisms 
(expressed as colony-forming unit (CFU)/100 μl lavage fluid). On day 10, the animals 
were bled retro-orbitally, humanely sacrificed, and similarly lavaged with subsequent 
processing for evaluation of vaginal fungal burden. Blood was processed for plasma 
and stored for drug level analyses. In addition for the first study with wild-type C. 
albicans, vaginal tissue was the collected and quick-frozen for drug level analyses.

**Drug level analyses.** Each sample was analyzed using LC/MS-MS with 
electrospray ionization, with quantification against an external calibration curve 
generated in the same matrix and using the signal response ratio between the sample 
and the internal standard. Vaginal tissue was homogenized in 50 mM PBS with a 
Brinkman Polytron PT10-35 homogenizer fitted with a 12 mm saw tooth generator, and 
then compound was extracted with methyl tert-butyl ether (MTBE). Compound was 
extracted from plasma samples with MTBE.

**Statistical analyses of fungal burden data.** Median values and range for 
fungal burden data were determined for each dose with 10 mice/group. Statistical
differences between dose groups were determined by the non-parametric Mann Whitney U test. Mean and standard deviation for plasma concentration were also determined for each dose group, with no further statistical analyses performed.
Results

VT-1161 MICs against sensitive and resistant C. albicans. VT-1161 was tested in micro-dilution assays against two fluconazole-sensitive C. albicans strains (MIC of 0.25 μg/ml for fluconazole) and 10 isolates taken from VVC patients that varied in their in vitro sensitivity to fluconazole (MIC ranged from 2 to 64 μg/ml, with a MIC50 of 8 μg/ml and a MIC90 of 64 μg/ml) (Table 1). For both sensitive strains and 8 of the 10 resistant clinical isolates, the MIC for VT-1161 was equal to or less than 0.015 μg/ml (the lowest concentration tested) (Table 1). The MICs for the other two fluconazole-resistant clinical isolates were 0.12 and 2 μg/ml. Therefore, the MIC50 and MIC90 values were ≤0.015 and 0.12 μg/ml, respectively, which were 500-fold more potent than the corresponding values for fluconazole.

Pharmacokinetics of VT-1161 in mice. The kinetics of VT-1161 were followed in both plasma and vaginal tissue after a single oral dose and in plasma after a single i.v. dose (Figure 1, Table 2). VT-1161 was quickly and efficiently absorbed after an oral dose into plasma (oral bioavailability of 73%) and rapidly and fully equilibrated into tissue; at all time points, the vaginal tissue level was at least 2-fold higher than plasma level (Figure 1). VT-1161 had a very long half-life after a single oral dose (>48 h); due to insufficient time points, an accurate oral half-life could not be determined. From the i.v. study, a large volume of distribution (1.4 kg/L) was calculated, suggesting that VT-1161 equilibrates into most body compartments and consistent with the measured high vaginal tissue levels.

The long half-life can be partly explained by two additional observations. VT-1161 was highly bound to mouse plasma protein (97.2%) and showed virtually no
metabolism after 65 min of incubation with mouse liver microsomes (Table 2).

Consistent with the long half-life of VT-1161, plasma concentrations accumulated after repeat dosing. For example, in the rat which displays an equally long oral half-life for VT-1161, when dosed once-daily for 7 days, $C_{\text{max}}$ and AUC exposures increased 6-7-fold when parameters derived from data taken on the first day were compared with those from the last day of dosing (E.P. Garvey and R.J. Schotzinger, unpublished data).

**VT-1161 efficacy against sensitive *C. albicans* in murine vaginitis.** VT-1161 was tested at 4, 10, and 25 mg/kg dosed orally once-daily for 4 days in a murine model of vaginal candidiasis using the *C. albicans* laboratory strain 3153A which was sensitive in vitro to both VT-1161 and fluconazole (Table 1). Fluconazole served as the positive control comparator and was dosed at 25 mg/kg orally once-daily for 4 days. Vehicle was dosed once-daily for 4 days as the negative control. Vaginal fungal burden was measured at both 1 and 4 days after treatment, and compound levels were measured four days after the last dose in both plasma and vaginal tissue samples (Table 3). The fungal burden in each treatment group was significantly decreased relative to vehicle control on both days of fungal burden analyses (all P values <0.0001, except for 10 mg/kg VT-1161 at 4 days after treatment, P = 0.0002).

Each treatment group showed essentially the same antifungal activity 1 day after treatment. There were no statistically different fungal burdens between any two treatment comparison (P values ranged between 0.40 and 0.78). Additionally, each group had approximately the same number of animals showing undetectable vaginal lavage CFUs (7/10 for 4 mg/kg VT-1161, 4/10 for 10 mg/kg VT-1161, 6/10 for 25 mg/kg VT-1161, and 5/9 for fluconazole). The fungal burdens 4 days after treatment showed
fungal "rebound" in the low- and mid-dose VT-1161 groups (Table 3; P values of 0.0185 and 0.0288 when comparing days 1 and 4 post-treatment data for the 4 and 10 mg/kg groups, respectively). Although fungal re-growth occurred, both doses were still superior to vehicle control (P values of <0.0001 and 0.0002, respectively). The effects with high-dose VT-1161 or fluconazole were sustained through the 4 days post-treatment (P value <0.0001 for each compared to vehicle, and 0.7394 and 0.6655, respectively, compared to 1-d post-treatments). The fungal re-growth in the low- and mid-dose VT-1161 groups was also reflected in the number of animals that had undetectable CFUs on day 4 after treatment (3/10 for low-dose and 1/10 for mid-dose VT-1161 versus 7/10 for high-dose VT-1161 and 7/9 for fluconazole).

Concentrations of drug in both plasma and vaginal tissue are also shown in Table 3. Based on the long half-life of VT-1161 in mice (Table 2), the high levels of VT-1161 were expected. Assuming that VT-1161 accumulated in mouse plasma similar to the accumulation previously observed in rat, we would expect a $C_{\text{max}}$ of approximately 25 $\mu$g/ml after the fourth and final dose of 10 mg/kg VT-1161. Using an estimated half-life of 48 h, plasma levels four days after the last dose would then be approximately 6 $\mu$g/ml (which is similar to the 4 $\mu$g/ml measured). Additionally, based on the short half-life of fluconazole in mice (19), the low levels remaining 4 days after the last dose were also expected. Both drugs fully equilibrated into vaginal tissue from the blood. The VT-1161 vaginal tissue data were consistent with the single-dose PK study, and the fluconazole data were consistent with a previous publication (20). Finally, it is noted that the strain of mouse used in the PK study (Balb/c) was different than the strain used in the model (CBA/J) and some components of PK may differ. However, it appears that
many PK parameters (good absorption, long half-life, and high tissue penetration) were similar between the two strains of mice.

**VT-1161 efficacy against resistant *C. albicans* in murine vaginitis.** Initially, a number of clinical isolates from VVC patients that had been tested *in vitro* (Table 1) were used in a pre-study to confirm infectibility *in vivo*. All isolates tested established robust infections to approximately the same extent as wild-type isolates. Subsequently, VT-1161 was tested at 25 mg/kg under the same protocol design as for the wild-type strain against three of the isolates (AR466-06, JJ330-05, and LP-1158-07) resistant to fluconazole (with MIC values of 64, 8, and 64 μg/ml, respectively). (For clarification, a recent EUCAST recommendation defined a fluconazole MIC of >4 μg/ml as being resistant (21).) Fluconazole at 25 mg/kg and vehicle were again the positive comparator and negative controls, respectively, for each isolate. Because of the large number of animals per study, two studies were conducted with two isolates tested in each study. The isolate AR466-06 was tested in each study to determine the reproducibility between studies. The CFUs on days 1 and 4 post-treatment and the plasma concentrations 4 days after treatment are shown in Table 4.

*Isolate AR466-06 (VT-1161-sensitive; Fluconazole-resistant).* The two independent studies with *C. albicans* AR466-06 gave reproducible results for both test compounds (Table 4). As expected based on its MIC of ≤0.015 μg/ml and the results above with the *C. albicans* 3153A strain, VT-1161 showed significant efficacy in both studies 1-d post-treatment compared to either vehicle control or fluconazole (P values ranging from 0.043 to 0.0019), and a trend toward sustained activity 4-d post-treatment compared to vehicle control (P values of 0.061 and 0.071) with continued efficacy.
relative to fluconazole (P values <0.0001 and 0.0003). Also, as expected based on its MIC of 64 μg/ml, fluconazole repeatedly had no effect on the vaginal lavage fungal burden measured at either time point in both studies (P values of 0.30 and 0.40 for day 1, respectively, and P values of 0.56 and 0.16 for 4-d post-treatment, respectively).

Isolate JJ330-05 (VT-1161-intermediate sensitive; Fluconazole-intermediate resistant). Based on C. albicans JJ330-05 being less susceptible to VT-1161 in vitro (MIC of 0.12 μg/ml), the in vivo antifungal activity of VT-1161 was in question prior to the study. VT-1161 retained significant suppression of vaginal lavage CFUs compared to vehicle control when measured at either time point (P values of 0.0003 and 0.0005 at 1 and 4-day post-treatment, respectively) (Table 4). Fluconazole's in vivo activity against this isolate was partial. It was active when fungal burden was measured 1-day post-treatment (P value <0.0001); however, when measure 4-d post-treatment, no antifungal activity was observed (P value 0.81). These data of partial activity were consistent with its in vitro MIC of 8 μg/ml, which is only slightly higher than the guidelines of >4 μg/ml being considered resistant (21). The statistical comparison between VT-1161 and fluconazole with this isolate was as expected based on the P values versus vehicle control - i.e., no difference was observed when comparing day-1 data (P value 0.24), and VT-1161 was superior when comparing day-4 data (P value 0.0021).

Isolate LP1158-07 (VT-1161-intermediate resistant; Fluconazole-resistant). Whereas no antifungal activity was expected with fluconazole with C. albicans LP1158-07 (MIC of 64 μg/ml), it was again uncertain if VT-1161 would retain in vivo activity with this isolate considering its in vitro activity (MIC of 2 μg/ml). Neither compound had
significant activity compared to either vehicle control or to each other (Table 4), with P values ranging from 0.065 to 0.30 for 5/6 of the comparisons. The comparison of the day-4 VT-1161 and vehicle control data showed that the vehicle control's CFU was significantly lower than VT-1161's CFU (P value 0.0011). However, given the equivalencies between fluconazole and vehicle control and VT-1161 and fluconazole at this time point, it is uncertain if this finding would be reproducible. Regardless, no analysis indicated that either compound had antifungal efficacy against this isolate.

Plasma drug concentrations. Plasma levels of VT-1161 and fluconazole in the studies of resistant isolates are shown in Table 4. All values are essentially within experimental error of each other and of the values determined in the first study using C. albicans 3153A (Table 3). This inter-study reproducibility is consistent with the relatively small standard deviations observed within each study, which reflect low inter-animal variability (Table 3 and 4). Together, these data indicate highly reproducible pharmacokinetics of both drugs.
Discussion

Vulvovaginal candidiasis is one of the most prevalent fungal infections for which patients seek medical treatment (1), and recurrent VVC is the most troublesome form of the disease in both discomfort (6) and difficulty of treatment (10). Although the exact prevalence is unclear (22), RVVC has been estimated to occur in as many as 9% of women living in Europe and the United States (23). The current standard of care for RVVC is maintenance fluconazole and has only a 42% success rate of maintaining a disease-free state for six months after treatment (10). New therapies are needed with greater efficacy. In addition, because of the need to treat over extended periods of time for this chronic condition, new therapies would ideally be dosed infrequently and have few if any side effects.

VT-1161 is a novel fungal CYP51 inhibitor that has completed Phase 2a clinical studies on both superficial and mucosal fungal infections (NCT01891305 and NCT01891331, respectively; www.clinicaltrials.gov). It was rationally designed to be highly selective relative to human CYP enzymes while maintaining potent inhibition of the fungal CYP target (16, 17). Its intrinsic in vitro antifungal potency against susceptible C. albicans was reproduced in this study where MIC was at or below the lowest concentration tested of 0.015 μg/ml). In addition, VT-1161 maintained this in vitro potency against 8 out of 10 clinical isolates that showed varying degrees of resistance to fluconazole (2-64 μg/ml). A much larger number of sensitive and resistant clinical isolates are required to fully characterize VT-1161 in vitro antifungal activity. However, these data coupled with MIC data from other laboratories (16, 17) and the biochemical data demonstrating nanomolar potency against the CYP51 target (17)
indicate that VT-1161 is one of the most potent CYP51 inhibitors of *C. albicans* yet described.

Coupled with this *in vitro* antifungal activity, VT-1161 showed excellent oral pharmacokinetics in the mouse; oral absorption was 73% with a high volume of distribution consistent with the high levels measured in vaginal tissue. Consistent with a long oral PK half-life (>48 h), VT-1161 was highly bound to mouse plasma protein and did not show any metabolism in mouse microsome incubations. These mouse PK and *in vitro* characteristics were consistent with those observed in the guinea pig (24) and in the rat, dog, and human (E.P. Garvey and R.J. Schotzinger, unpublished data).

Given its intrinsic antifungal potency, safety, and pharmacokinetics, VT-1161 is an ideal candidate for treating a number of diverse Candidal infections (e.g., invasive, mucosal, and cutaneous). Specifically, it was a prime candidate to test in a stringent murine model of vaginal candidiasis where infection is allowed to establish itself for 3 days prior to treatment (18). In the initial study using a fluconazole-sensitive strain of *C. albicans*, oral doses as low as 4 mg/kg VT-1161 were highly statistically efficacious in reducing fungal burdens in vaginal lavage samples on both 1 and 4 days after the last treatment. The two lower dose groups of VT-1161 showed some rebound of fungal growth on the day-4 evaluation. However, even with these increases, the values were highly statistically suppressed relative to vehicle controls. The dose of 25 mg/kg VT-1161 showed no re-growth at day 4 relative to day 1, either in comparing day 1 and 4 post-treatment mean values for fungal burden or in comparing number of animals that had undetectable fungus in lavage samples. Additionally, when directly comparing at the same dose of 25 mg/kg, VT-1161 was equivalent to fluconazole in this model, again
both in comparison of mean values for fungal burden and in number of animals that had undetectable fungus.

Plasma concentrations of VT-1161 after 4 days of dosing and another 4 days off drug were similar to expectations based on the approximate half-life of each drug (i.e., relatively high for VT-1161 and low for fluconazole). VT-1161 concentrations were also determined in vaginal tissue and at each dose showed full equilibration between plasma and vagina, consistent with the single dose PK study and high volume of distribution.

VT-1161 largely retained in vitro activity against fluconazole-resistant clinical isolates, and we hypothesized that VT-1161 would suppress fungal growth in vivo against isolates that were fully sensitive to VT-1161. However, it was unclear if VT-1161 could suppress in vivo growth of isolates less susceptible to it. As would be predicted based on MIC values reflecting full sensitivity to VT-1161 ($\leq 0.015 \mu g/ml$) and full resistance to fluconazole ($64 \mu g/ml$), VT-1161 was reproducibly efficacious in suppressing in vivo growth of the AR466-06 isolate, whereas fluconazole was inactive.

When the JJ330-05 isolate with "intermediate" susceptibility was used, VT-1161 (MIC = 0.12 $\mu g/ml$) remained highly efficacious relative to vehicle control. Fluconazole (MIC = 8 $\mu g/ml$) had "partial" in vivo activity insofar as being able to suppress growth 1 day after treatment, but did not show sustained effects 4 days after treatment. Finally, neither inhibitor had any effect on growth measured either 1 or 4 days after treatment with the isolate LP-1158-07 (MIC = 2 and 64 $\mu g/ml$ for VT-1161 and fluconazole, respectively).

The plasma levels of both drugs were very similar in all of these studies with relatively low inter-animal variability, demonstrating reproducibility of their oral pharmacokinetics.
The above data can be used to predict what drug concentrations should be targeted to achieve VT-1161 *in vivo* efficacy. For invasive fungal infections, it is widely accepted that plasma concentration of free drug is the most relevant value and that area under the curve (AUC) is the most relevant PK parameter (25). If free drug is also true for mucosal infections such as VVC, then VT-1161 binding to mouse plasma (97.2%) needs to be considered in analyzing these data. Because only single point plasma levels were determined in these studies, AUC values could not be calculated. During the four days of dosing, VT-1161 accumulated to reach a $C_{\text{max}}$ after the last dose, and then was slowly eliminated. Using the approximate half-life of VT-1161 in the mouse of 48 h, the $C_{\text{max}}$ after the last dose is estimated to be ~4 times higher than the concentration measured 4 days after. This estimated range of total plasma concentration can be multiplied by 0.029 to obtain an estimate of free drug, and both total and free levels can then be compared to the intrinsic MIC potency for each isolate examined in these studies.

Using this approach, both total and free plasma concentrations of VT-1161 were above the *in vitro* MIC values for wild-type, AR466-06, and JJ330-05 isolates, consistent with the antifungal efficacy observed in those studies, but not distinguishing between total and free VT-1161 concentrations. Whereas the concentration range of total VT-1161 in the LP-1158-07 study was well above the MIC against the isolate (2 μg/ml), the range of free VT-1161 was below, and thus correlated with the lack of efficacy observed with LP-1158-07. Additionally, the predicted total vaginal tissue levels were well above the MIC of 2 μg/ml for this insensitive isolate. Therefore, taken together, these data...
indicate that free VT-1161 in the plasma was the best predictor of efficacy in this murine model of vaginal candidiasis.

In summary, the in vivo efficacy of VT-1161 in the murine model of vaginal candidiasis provides strong support toward clinical development of this agent in treating VVC. Likewise, the excellent in vitro and in vivo activity profile that VT-1161 displayed against resistant C. albicans bodes well for its ability to treat infections caused by isolates that are resistant to currently approved CYP51 inhibitor drugs. The strong correlate of efficacy to free drug in plasma based on MIC allows for possible monitoring for more accurate dosing that may result in even fewer treatments. Furthermore, because of the high selectivity of VT-1161 for its fungal CYP target relative to off-target human CYP enzymes (17), few if any side effects are expected. Finally, because of the long half-life of VT-1161, infrequent dosing regimens such as once-weekly maintenance dosing after an initial loading dose would be attractive regimens for such chronic infections as RVVC. Given all of the above, a Phase 2b study of VT-1161 treatment of RVVC using a loading dose/maintenance dose regimen is now in progress (NCT02267382; www.clinicaltrials.gov).
Acknowledgements

All LC/MS-MS drug level measurements were done at OpAns, LLC (Durham, NC). All work described in this manuscript was supported by Viamet Pharmaceuticals, Inc (Durham, NC 27703).
References


**Figure Legend**

**Fig 1** VT-1161 Oral and IV Pharmacokinetics in the Mouse. A single dose of VT-1161 was given by oral gavage at 5 (oral) and 2 (i.v.) mg/kg both in 20% Cremaphor EL in female Balb/c mice. Plasma and vaginal tissue samples in the oral study and plasma samples in the i.v. study were collected up to 48 h (N=3 animals per time point). Data points are mean values with error bars representing the standard deviation.
**Fig 1** VT-1161 Oral and IV Pharmacokinetics in the Mouse. A single dose of VT-1161 was given by oral gavage at 5 (oral) and 2 (i.v.) mg/kg both in 20% Cremaphor EL in female Balb/c mice. Plasma and vaginal tissue samples in the oral study and plasma samples in the i.v. study were collected up to 48 h (N=3 animals per time point). Data points are mean values with error bars representing the standard deviation.
Table 1. VT-1161 and Fluconazole MICs against clinical isolates from VVC/RVVC patients.

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<th>MIC, μg/ml</th>
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<td>3153A (lab strain)</td>
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<td>MR700-13</td>
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Table 2. *In vivo* and *in vitro* pharmacokinetic parameters for VT-1161 in the mouse.

<table>
<thead>
<tr>
<th></th>
<th>2 mg/kg i.v.</th>
<th>5 mg/kg oral</th>
<th>Plasma Protein Binding (%)</th>
<th>Liver Microsomal Stability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>~t_{1/2} (h)</td>
<td>11 (2)</td>
<td>&gt;48¹</td>
<td>73</td>
<td>100%⁵</td>
</tr>
<tr>
<td>V_{ss} (L/kg)</td>
<td>1.4 (0.2)</td>
<td>0.8 (0.2)</td>
<td>97.2 (0.4)</td>
<td></td>
</tr>
<tr>
<td>C_{max} (μg/ml)</td>
<td>0.8 (0.2)</td>
<td>33 (6)</td>
<td>97.2 (0.4)</td>
<td></td>
</tr>
<tr>
<td>AUC_{0-48h} (h*μmol/ml)</td>
<td>33 (6)</td>
<td>&gt;48¹</td>
<td>97.2 (0.4)</td>
<td></td>
</tr>
<tr>
<td>F (%)</td>
<td>73</td>
<td>73</td>
<td>100%⁵</td>
<td></td>
</tr>
</tbody>
</table>

All values are means (standard deviation). ¹t_{1/2} value could not be accurately determined due to the lack of data at extended time periods. ²All time points showed >92% remaining with the average value being 98% remaining to the zero time point.
Table 3. VT-1161 Efficacy in Murine Vaginal Candidiasis with Wild-type *C. albicans*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Post-Treatment CFU&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Compound Level&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 4</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1290 (926-2893)</td>
<td>15166 (12875-23292)</td>
</tr>
<tr>
<td>FLU (25 mg/kg)</td>
<td>0 (0-70) P&lt;0.0001</td>
<td>0 (0-70) P&lt;0.0001</td>
</tr>
<tr>
<td>VT (4 mg/kg)</td>
<td>0 (0-13) P&lt;0.0001</td>
<td>2790 (0-3739) P&lt;0.0001</td>
</tr>
<tr>
<td>VT (10 mg/kg)</td>
<td>25 (0-143) P&lt;0.0001</td>
<td>2790 (18-8500) P=0.0002</td>
</tr>
<tr>
<td>VT (25 mg/kg)</td>
<td>0 (0-140) P&lt;0.0001</td>
<td>0 (0-108) P&lt;0.0001</td>
</tr>
</tbody>
</table>

FLU = fluconazole. <sup>1</sup>Median (interquartile range) CFU/100 μl lavage fluid either 1 or 4-d post-treatment. P values compared to vehicle using Mann-Whitney U test. Bolded values indicate significance. <sup>2</sup>Mean (standard deviation) level 4-d post-treatment.
**Table 4. VT-1161 Efficacy in Murine Vaginal Candidiasis using clinical isolates C. albicans with Reduced Susceptibility to Fluconazole.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Post-Treatment CFU&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Plasma Compound Level (&lt;i&gt;μg/ml&lt;/i&gt;)&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 4</td>
</tr>
</tbody>
</table>

**AR466-06 1st Study**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1300 (868-3758)</td>
<td>825 (15-4413)</td>
</tr>
<tr>
<td>FLU (25 mg/kg)</td>
<td>1000 (583-1488)</td>
<td>1100 (525-2813)</td>
</tr>
<tr>
<td>VT (25 mg/kg)</td>
<td>142 (38-298)</td>
<td>52 (0-136)</td>
</tr>
<tr>
<td></td>
<td>P=0.3042</td>
<td>P=0.0610</td>
</tr>
<tr>
<td>P(FLU)=0.0019</td>
<td>P(FLU)&lt;=0.0001</td>
<td>16 (5)</td>
</tr>
</tbody>
</table>

**AR466-06 2nd Study**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>3342 (1563-14250)</td>
<td>290 (73-1475)</td>
</tr>
<tr>
<td>FLU (25 mg/kg)</td>
<td>1775 (1198-6142)</td>
<td>1075 (450-2388)</td>
</tr>
<tr>
<td>VT (25 mg/kg)</td>
<td>450 (66-1788)</td>
<td>65 (8-130)</td>
</tr>
<tr>
<td></td>
<td>P=0.4040</td>
<td>P=0.0713</td>
</tr>
<tr>
<td>P(FLU)=0.0089</td>
<td>P(FLU)=0.0003</td>
<td>14 (4)</td>
</tr>
</tbody>
</table>

**JJ330-05**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>47916 (35250-166250)</td>
<td>11250 (4288-26500)</td>
</tr>
<tr>
<td>FLU (25 mg/kg)</td>
<td>1875 (950-4500)</td>
<td>10300 (5817-40458)</td>
</tr>
<tr>
<td>VT (25 mg/kg)</td>
<td>4583 (863-10625)</td>
<td>52 (164-2400)</td>
</tr>
<tr>
<td></td>
<td>P=0.8111</td>
<td>P=0.0713</td>
</tr>
<tr>
<td>P(FLU)=0.0001</td>
<td>P(FLU)=0.0005</td>
<td>16 (3)</td>
</tr>
</tbody>
</table>

**LP1158-07**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>19250 (9300-213500)</td>
<td>4500 (1850-8033)</td>
</tr>
<tr>
<td>FLU (25 mg/kg)</td>
<td>12000 (4375-28667)</td>
<td>7000 (3250-19500)</td>
</tr>
<tr>
<td>VT (25 mg/kg)</td>
<td>7000 (3500-13000)</td>
<td>18000 (11625-39167)</td>
</tr>
<tr>
<td></td>
<td>P=0.0854</td>
<td>P=0.0011 (inferior)</td>
</tr>
<tr>
<td>P(FLU)=0.2395</td>
<td>P(FLU)=0.0021</td>
<td>14 (4)</td>
</tr>
</tbody>
</table>

---

<sup>1</sup>Median (interquartile range) CFU/100 μl lavage fluid on 1 or 4-d post-treatment. P value compared to vehicle; P(FLU) compared to FLU. P values determined using Mann-Whitney U test. Bolded values indicate significance.

<sup>2</sup>Mean (standard deviation) level 4-d post-treatment.