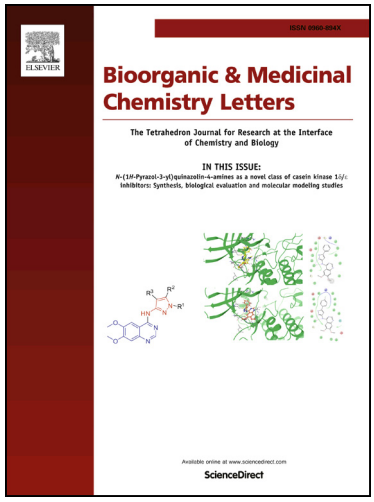

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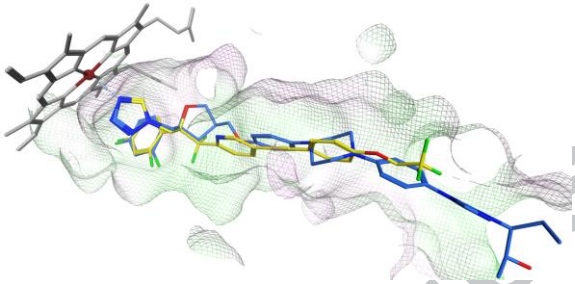

Design and Optimization of Highly-Selective, Broad Spectrum Fungal CYP51 Inhibitors

Christopher M. Yates*, Edward P. Garvey, Sammy R. Shaver, Robert J. Schotzinger, and William J. Hoekstra

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Abstract: While the orally-active azoles such as fluconazole and posaconazole are effective antifungal agents, they potently inhibit a broad range of off-target human cytochrome P450 enzymes (CYPs) leading to various safety issues (e.g., drug-drug interactions, liver, and reproductive toxicities). Recently we described the rationally-designed, antifungal agent VT-1161 that is more selective for fungal CYP51 than related human CYP enzymes such as CYP3A4. Herein, we describe the use of a homology model of *Aspergillus fumigatus* to design and optimize a novel series of highly selective, broad spectrum fungal CYP51 inhibitors. This series includes the oral antifungal VT-1598 that exhibits excellent potency against yeast, dermatophyte, and mold fungal pathogens.

Metalloenzymes present a major challenge to the development of potent and selective inhibitors due to the high homology common in the area around the metal. Many metalloenzyme inhibitors consist of two chemical components: the metal-binding group (MBG), the portion of the inhibitor designed to bind to the metal, and the scaffold, the portion of the inhibitor recognized by the amino acid residues that form the substrate-binding site of the metalloenzyme. The MBG interaction is a key component to developing potent inhibitors of metalloenzymes but if the metal/MBG interaction is too strong, unintended interactions with related metalloenzymes can occur as well. The approach of attenuating the metal/MBG interaction in conjunction with optimization of the scaffold has been successful in the development of potent and selective cytochrome P450 enzyme inhibitors, [1,2] including fungal CYP51 (lanosterol 14 α-demethylase).[1]

Historically the azole class of antifungal drugs have inhibited fungal CYP51 activity through strong, reversible binding to the heme-iron located in the enzyme active site utilizing 1-imidazole and 1-(1,2,4-triazole) MBGs.[3] Unfortunately, while the use of these MBGs has provided potent antifungal drugs, these drugs are hampered by liver and reproductive toxicities and drug-drug interactions.[4,5] Significant advances have been made in the discovery of selective fungal CYP51 inhibitors through utilizing a 1-tetrazole as a preferred MBG.[1] This led to the antifungal compound VT-1161 (Figure 1) which has demonstrated excellent yeast and dermatophyte CYP51 inhibitory potency while maintaining selectivity towards human CYP51 and human hepatic CYP enzymes.[6] VT-1161 has recently completed two separate Phase 2b studies for treatment of onychomycosis (NCT02267356) and recurrent vulvovaginal candidiasis (NCT02267382).

Figure 1. Clinical and marketed antifungal agents.

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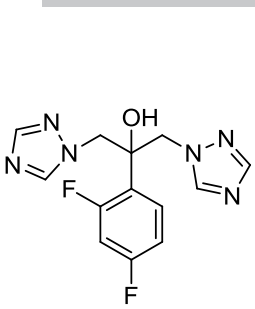
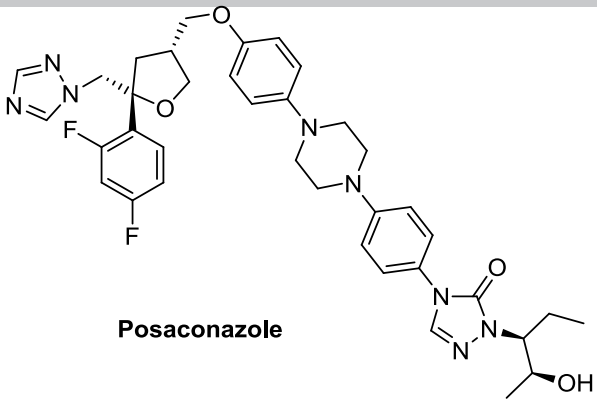
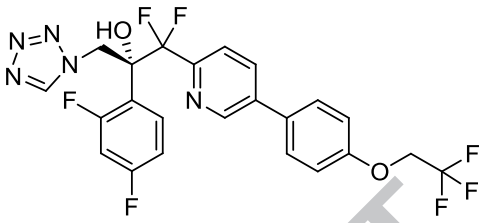


Fluconazole



Posaconazole

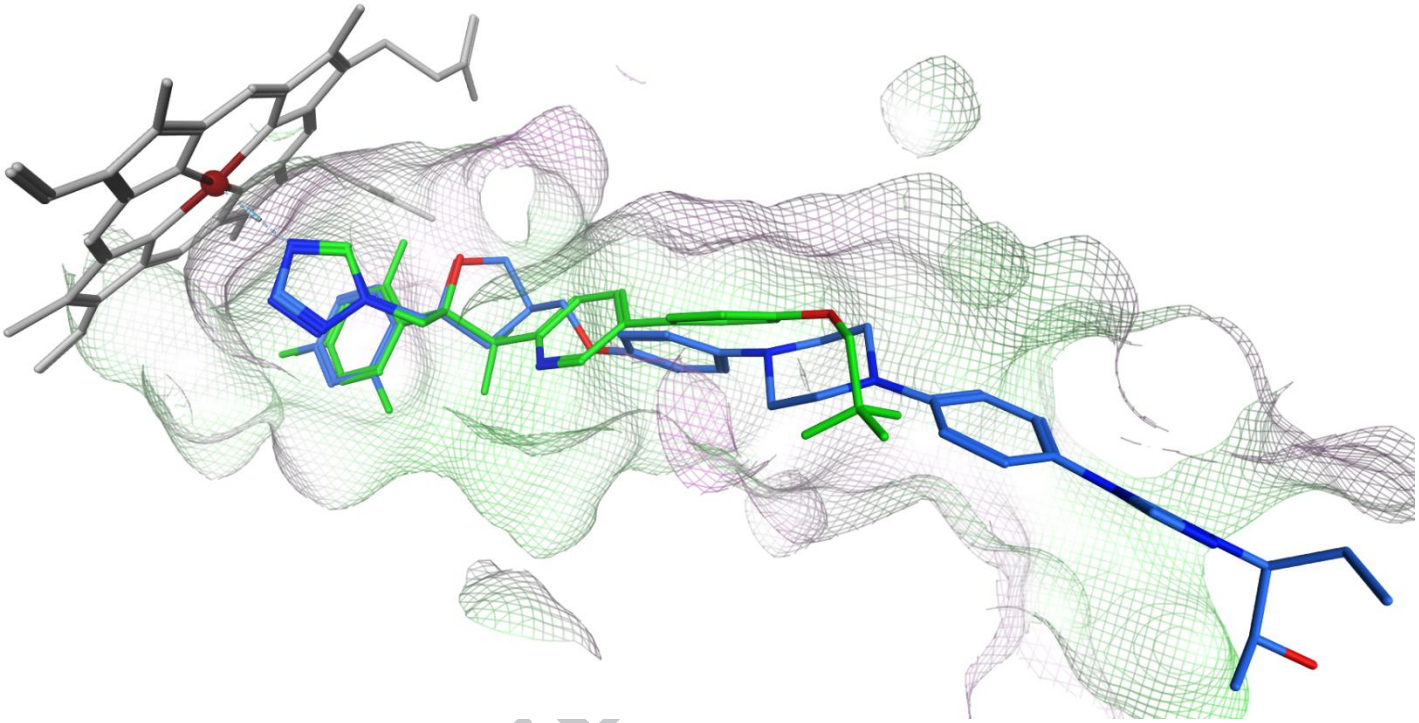


VT-1161

Although many fungal infections are treated with acute therapy, many invasive fungal infections persist and require chronic therapy for weeks, months, or even years. Chronic fungal infections such as cryptococcal[7] or coccidioidal[8] meningitis are life-threatening, while other infections such as chronic pulmonary aspergillosis[9] and refractory pneumonia caused by endemic fungi[8] seriously impact quality of life. In addition, infections such as chronic mucocutaneous candidiasis are less serious but nonetheless require chronic therapy.[10] An ideal drug for the long-term treatment of such infections would have a broad spectrum that covers yeast, mold, and endemic fungi, would have a patient-friendly dosing regimen (e.g., oral once-daily), and would be very safe with few if any side effects and drug-drug interactions. Although many efficacious antifungal drugs have been approved, no current drug has all the above attributes. For example, many of the approved azoles, such as posaconazole (Figure 1), have sufficiently broad coverage of fungal pathogens, but are highly prone to side effects due to inhibition of human CYP enzymes.[4,5] These side effects not only limit patient tolerability towards therapy, but they can also limit the dose of drug and thereby limit efficacy in treating these very serious infections.

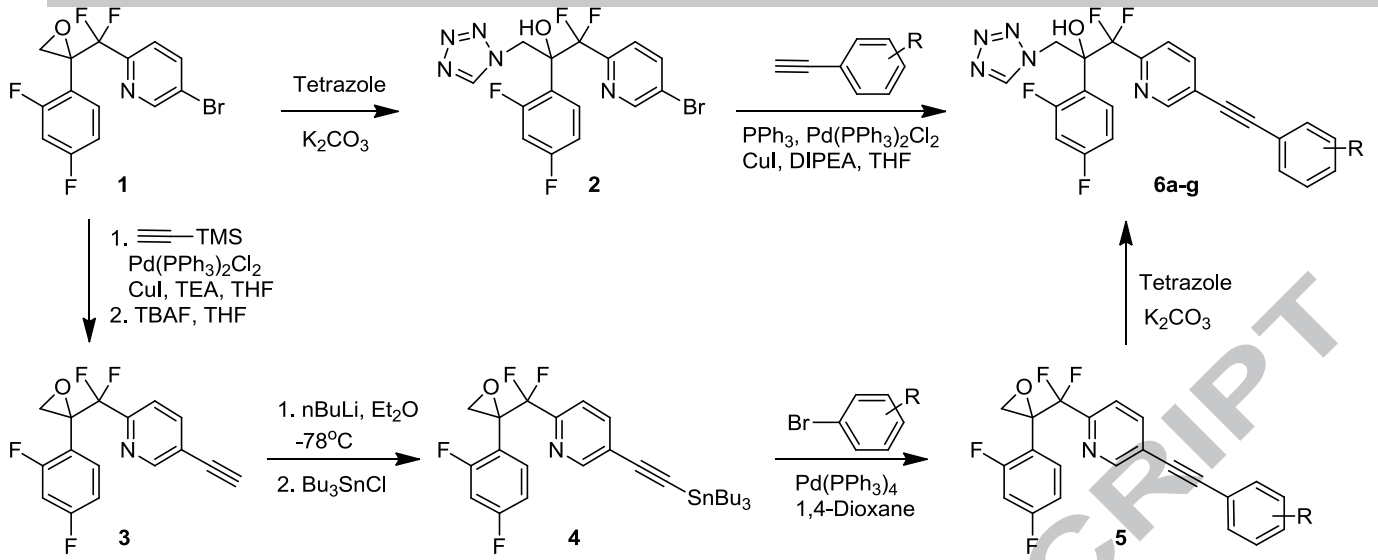
To aid in the development of a broad spectrum fungal CYP51 inhibitor, *A. fumigatus*-CYP51 was chosen as an initial target due to the lack of mold activity in the previous discovery program. As there were no *A. fumigatus* crystal structures available to provide structural guidance upon initiation of this program, we created a series of homology models suitable for guiding our medicinal chemistry efforts. The homology models were created using Molecular Operating Environment (MOE).[11,12,13] To enable development of a useful homology model, a series of models were constructed using seven protein crystal structures available from the Protein Data Bank (PDB codes: 2BZ9, 3G1Q, 3K1O, 2X2N, 3KHM, 2WV2, 1EA1) as templates.[14] The preliminary homology models were evaluated by docking a series of internal azole compounds, as well as fluconazole and posaconazole.[15] The models were rank ordered by their performance in correctly predicting relative activity, using binding affinity as a surrogate. As compounds were synthesized and assayed, the accuracy of the models was continually monitored and refined. Many of the models constructed from templates with no ligand or small ligands were not useful due to the access channel being collapsed between the active site and the surface of the protein. The models chosen for continued development were constructed using 3K1O, a *Trypanosoma cruzi* crystal structure with posaconazole bound in the active site.[16,17]

Figure 2. Overlay of VT-1161 (green) and posaconazole (blue) in an *A. fumigatus*-CYP51 homology model. The grid lines show the active site near the near the heme-iron and the access channel towards the surface of the protein. The grid colors, purple and green representing hydrophilic and lipophilic, respectively, show the predominantly lipophilic characteristics of the CYP51 access channel. The shorter VT-1161 is not able to reach the bend in the channel and fill the lipophilic space near the entrance.



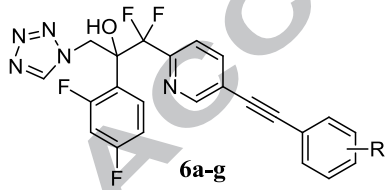
Our previous lead antifungal compound, VT-1161, was utilized for initial evaluation of the *A. fumigatus* homology model and to aid in the design of a new series of compounds for synthesis. When comparing the docked views of VT-1161 in the *Candida albicans* crystal structure and the new *A. fumigatus* homology model, it could be seen that the linear portion of the *A. fumigatus* access channel is extended before it bends out to the surface of the protein. This was further confirmed by docking posaconazole in the *A. fumigatus* model and overlaying it with the VT-1161 docking (Figure 2). It was hypothesized that extended analogs would have improved interactions with the hydrophobic access channel. A series of extended analogs with varied linkers, flexibility, and trajectories were designed and docked to provide prioritization for synthesis. The ethyne series showed significant potential in the homology model and was therefore chosen for initial evaluation. A series of aryl ethyne analogs, **6a-g**, maintaining several of the key components developed in VT-1161, including the 1-tetrazole MBG, was prepared using Sonogashira or Stille couplings (Scheme 1; Table 1).[18]

Scheme 1



The 4-fluorophenyl ethyne **6a** showed promise in regards to maintaining potent yeast activity but the larger lipophilic groups, demonstrated by **6b** and **6c**, showed a drop in antifungal activity. Antifungal activity was also diminished when the halo group was shifted to the meta-position of the terminal phenyl ring, **6d** and **6e**. The extended ether and aniline based compounds **6f** and **6g** showed modest anti-*A. fumigatus* activity against mold while retaining the strong anti-yeast activity, but **6g** also showed a decrease in selectivity ratio in regards to inhibition of CYP3A4. Previous experience in the development of VT-1161 and historical data in the azole class of antifungal agents have demonstrated that the active site of CYP51 is sensitive in regards to stereochemistry near the heme-iron. To test the impact of chirality of the ethyne series, example **6a** was selected for resolution by chiral HPLC. The resulting enantiomers were submitted for screening to furnish the modest boost in antifungal activity of **6a(+)** against *A. fumigatus* (**6a(-)** was inactive against both *C. albicans* and *A. fumigatus*, data not shown).

Table 1. Antifungal Activity and CYP3A4 Potency of Aryl Ethyne Inhibitors.



Compound	R	*C. albicans* MIC[a]	*A. fumigatus* MIC[b]	CYP3A4 IC_{50}[c]
6a	4-F	<0.001	>64	100
6a(+)	4-F	<0.001	16	198
6b	4-Cl	0.004	>64	>200
6c	4-CF_3	0.016	>64	>200
6d	3-F	0.004	>64	>200
6e	3-Cl	0.031	>64	>200
6f	4-OCH_2CF_3	<0.001	64	60

6g	4-$NHCH_2CF_3$	<0.001	4	10
Posaconazole	-	0.015	0.062	0.075
Fluconazole	-	0.5	>64	32
VT-1161	-	<0.001	>64	65

a. Minimum concentration that achieved 50% inhibition of fungal growth; MIC units in µg/mL.[19] b. Minimum concentration that achieved 100% inhibition of fungal growth; MIC units in µg/mL.[19] c. Inhibition of CYP3A4 measured biochemically using microsomes obtained from pooled human hepatocytes; IC_{50} units in µM.[20]

Further homology modeling of **6a(+)** showed the compound filled the access channel in a similar manner to VT-1161 but the extended analogs, **6f** and **6g,** were able to follow the bend and begin to fill the lipophilic channel towards the surface (Figure 3). Encouraged by the activity and potential of the ether series to both maintain selectivity and improve activity against *A. fumigatus*, a series of benzyl ethers was synthesized to extend towards the surface. The compounds were synthesized in modest yields by alkylating 4-bromophenol with benzyl bromides. The resulting aryl bromides were coupled with **4** and the epoxide opening was performed with tetrazole to obtain the desired targets **7a-g** in a manner analogous to Scheme 1.[18]

Figure 3. CYP51 active site view of **6f** (yellow) docked into *A. fumigatus*-CYP51 homology model and overlaid with posaconazole (blue).

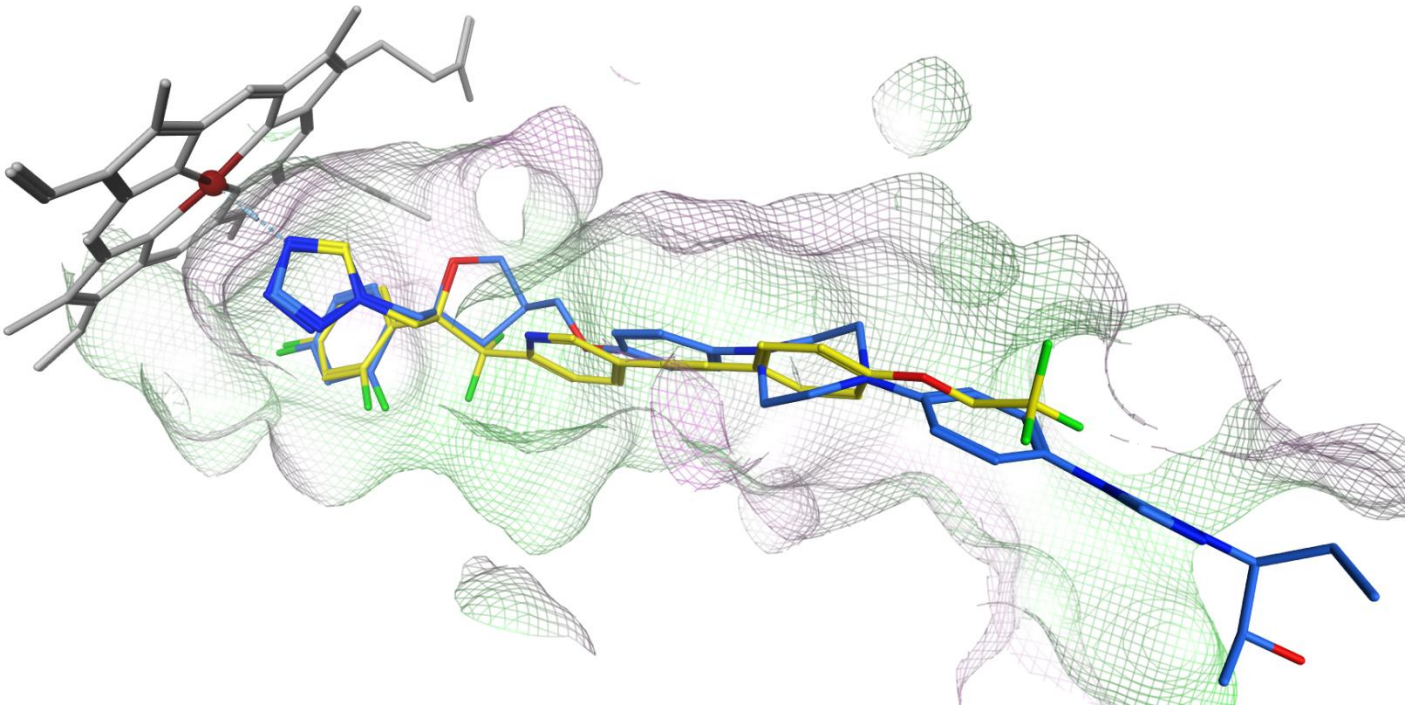
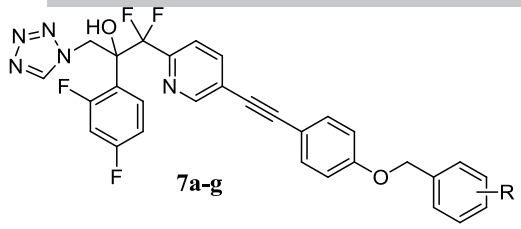


Table 2. Antifungal Activity and CYP3A4 Potency of Benzyl Ethers.



7a-g

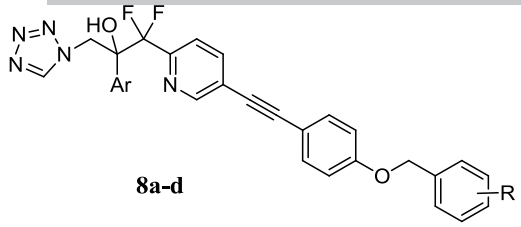
Compound	R	*C. albicans* MIC[a]	*A. fumigatus* MIC[b]	CYP3A4 IC_{50}[c]
7a	4-OCF_3	0.25	>64	>200
7b	4-CF_3	0.125	>64	>200
7c	4-CONHMe	0.031	>64	122
7d	4-F	0.0625	32	>200
7e	4-CHF_2	0.0625	4	>200
7e(+)	4-CHF_2	0.031	2	>200
7f	4-CN	0.031	8	>200
7f(+)	4-CN	0.0078	0.25	>200
7g	3-CN	0.016	>64	>200

a. Minimum concentration that achieved 50% inhibition of fungal growth; MIC units in µg/mL.[19] b. Minimum concentration that achieved 100% inhibition of fungal growth; MIC units in µg/mL.[19] c. Inhibition of CYP3A4 measured biochemically using microsomes obtained from pooled human hepatocytes; IC_{50} units in µM.[20]

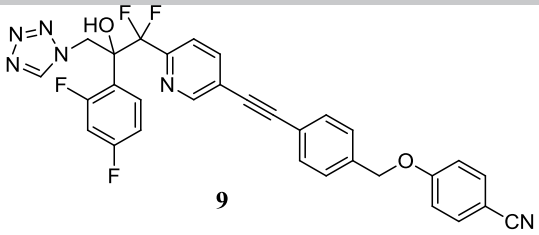
A variety of moieties were explored with the primary focus on electron withdrawing groups in the 4-position of the terminal aryl group near the surface due to size limitations in the access channel and to aid in stabilizing the benzyl group from metabolism (Table 2). The 4-trifluoromethyl ether (**7a**), 4-trifluoromethyl (**7b**), and 4-acetamide (**7c**) showed no antifungal activity against *A. fumigatus*. The 4-fluoro (**7d**), 4-difluoromethly (**7e**), and 4-cyano (**7f**) compounds showed promise as racemates, therefore the two more potent inhibitors, **7e** and **7f,** were resolved as single isomers to provide compounds with improved activity, **7e(+)** and **7f(+)**. Compound **7f(+)** demonstrated significant improvement in anti-*A. fumigatus* activity. Interestingly, although the homology modeling showed space for substitution near the surface of the protein, shifting the cyano to the meta position, **7g**, led to a loss of *A. fumigatus* antifungal activity. The series overall maintained good anti-yeast activity and excellent selectivity in regards to CYP3A4.

Since **7f** showed promising activity, additional analogs were synthesized maintaining the cyano portion of the molecule (Table 3). Unfortunately, altering the aryl group in **8** led to loss of anti-mold activity, as exemplified by **8c** and **8d**. A variety of moieties were explored in combination with cyano on the terminal phenyl group, with only the 3-fluoro, 4-cyano benzyl ether **8a** showing promising activity; however, its resolved enantiomer **8a(+)** fell short of the activity of **7f(+)**. We also experimented with the inverse ether **9**, but this led to diminished activity.

Table 3. Activity profile of **7f** analogs.



8a-d



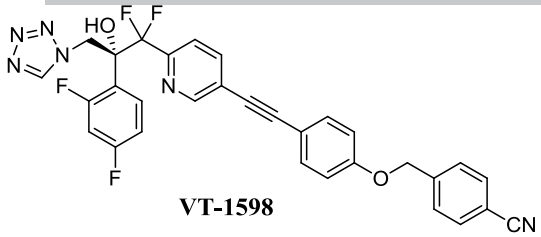
9

Compound	R	Ar	*C. albicans* MIC[a]	A. fumigatus MIC[b]	CYP3A4 IC_{50}[c]
8a	3-F, 4-CN	2,4-DiF	0.031	1	>200
8a(+)	3-F, 4-CN	2,4-DiF	0.004	0.5	>200
8b	2-F, 4-CN	2,4-DiF	0.16	>64	>200
8c	4-CN	2-F, 4-Cl	0.031	>64	>200
8d	4-CN	2,5-DiF	0.016	>64	>200
9	-	-	0.031	>64	>200

a. Minimum concentration that achieved 50% inhibition of fungal growth; MIC units in μg/mL.[19] b. Minimum concentration that achieved 100% inhibition of fungal growth; MIC units in μg/mL.[19] c. Inhibition of CYP3A4 measured biochemically using microsomes obtained from pooled human hepatocytes; IC_{50} units in μM.[20]

Further evaluation of the representative ethynyl ether VT-1598 (**7f(+)**) demonstrated a marked affinity for *A. fumigatus*-CYP51 (K_d = 13 nM), and a co-crystal structure with VT-1598 and *A. fumigatus* CYP51B has been obtained.[21,22] In an initial study of the breadth of intrinsic antifungal activity, VT-1598 had broad coverage, similar to posaconazole (Table 4). Whereas posaconazole was slightly more potent against mold isolates, VT-1598 was slightly more potent against yeast isolates, and both were much more potent than fluconazole against all species tested. In terms of selectivity of inhibiting fungal CYP51, when tested against a panel of key human CYP450 enzymes, VT-1598 showed weak or no inhibition up to the top concentration tested (Table 5). In contrast, both posaconazole and fluconazole showed inhibition potencies that have been proven to be clinically relevant in terms of unwanted side effects. For example, these inhibition potencies against CYP2C9, 2C19, and 3A4 can be directly correlated with drug-drug interactions.[6] Finally, VT-1598 had robust pharmacokinetics when dosed orally in mice (5 mg/kg).[23] The mean time to reach maximum measured plasma concentration (T_{max}), C_{max}, AUC_{last}, and $t_{1/2}$ values were 4 hours, 2.5 μg/mL, 67 μg·hr/mL, and 24 hours, respectively. VT-1598 concentrations were also measured in disease relevant tissues, brain, lung, and kidney, to determine tissue levels. Relative to plasma, VT-1598 exposures were higher in kidney with C_{max} and AUC_{48} values 270% and 268% of plasma, respectively. In the lung, C_{max} and AUC_{48} values were 116% and 94% of plasma, respectively, and in the brain, C_{max} and AUC_{48} values were 58% and 65% of plasma, respectively. The $t_{1/2}$ for each tissue was similar to plasma half-life, ranging from 16 to 19 hours. Given its overall profile, VT-1598 has been progressed into advanced antifungal screening and *in vivo* models,[24,25] which will be the subject of future publications.

Table 4. Broad Spectrum Antifungal Activity of VT-1598 (**7f(+)**).



VT-1598

Compound	*C. albicans* MIC[a]	*C. glabrata* MIC[a]	*C. neoformans* MIC[a]	*A. fumigatus* MIC[b]	*A. flavus* MIC[b]	*T. rubrum* MIC[a]
VT-1598	0.0078	0.12	0.0019	0.25	0.5	0.25
Posaconazole	0.015	0.12	0.0078	0.062	0.25	0.25
Fluconazole	0.5	8	0.25	>64	>64	16

a. Minimum concentration that achieved 50% inhibition of fungal growth; MIC units in μg/mL.[19] b. Minimum concentration that achieved 100% inhibition of fungal growth; MIC units in μg/mL[19]

Table 5. Biochemical Inhibition of Human CYP Enzymes by Fungal CYP51 Inhibitors.

	IC_{50}, μM[19]							
Human CYP	**2C9**	**2C19**	**3A4**	**11B1**	**11B2**	**17lyase**	**17OHase**	**19**
VT-1598	>200	138	>200	>100	>100	38	>100	>100
Posaconazole	109	7.2	0.075	0.31	0.021	0.042	1.8	8.4
Fluconazole	34	13	32	30	13	>200	>200	22

The development of homology models for the *A. fumigatus*-CYP51 has guided our effort in the development of a novel, 1-tetrazole-based series of antifungal compounds. Building off the potent and selective antifungal VT-1161, a series of modifications led to the discovery of a promising ethynyl ether series that has provided compounds with strong anti-*A. fumigatus* activity in addition to retaining high potency against yeast and dermatophytes. VT-1598 is a promising drug candidate which has broad antifungal activity in combination with an excellent selectivity and pharmacokinetic profile. As such, VT-1598 may possess the ideal attributes for a safe, broad-acting antifungal to treat serious chronic invasive fungal infections.

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13. The homology models were prepared by selecting a protein crystal structure available from the RCSB Protein Data Bank, aligning the selected pdb file with the sequence of human CYP51A (UniProtKB, Q16850), then running the homology modeler program within MOE using Amber 99 as the forcefield while selecting the heme and ligand atoms as an environment for induced fit. 10 intermediate models are created with sidechain sampling at 300K followed by final scoring using the GB/VI (generalized Born/volume integral) forcefield at a RMS gradient of 0.5. Ramachandran plot analysis of the final homology model shows 8 residues as outliers (1.68% of 475 residues).
14. RCSB Protein Data Bank http://www.rcsb.org/pdb/home
15. The binding energies in the optimized homology model for fluconazole, VT-1161, and posaconazole were -9.43, -11.67, and -14.98, respectively. The binding energy for VT-1598 was -12.72 kcal/mol.
16. The structure of posaconazole represented in the published pdb file, 3K1O, is incorrect. The correct structure of posaconazole was used in all homology work described herein and was based upon the following reference: Bennett, F; Saksena, A.K.; Lovey, R.G.; Liu, Y.T; Patel, N.M; Pinto, P.; Pike, R.; Jao, E.; Girijavallabhan, V.M. Ganguly, A.K.; Loebenberg, D.; Wang, H.; Cacciapuoti, A.; Moss, E.; Menzel, F.; Hare, R.S.; Nomeir, A. Hydroxylated analogues of the orally active broad spectrum antifungal, Sch 51048 (1), and the discovery of posaconazole [Sch 56592; 2 or (S,S)-5], *Bioorg. Med. Chem. Lett*. **2006**, *16(1)*, 186-190.
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18. Hoekstra, W.J.; Yates C.M. PCT Int. Appl. **2013**, WO2013090210.
19. Minimum inhibitory concentrations (MIC) for standard ATCC isolates were determined under the CLSI guidelines M27-A3 (with yeast endpoints being 50% inhibition of growth at 48 hours) and M38-A2 (with mold endpoints being 100% inhibition of growth at 48 hours and dermatophyte endpoints being 100% inhibition at 168 hours) (Eurofins Panlabs, Taiwan).
20. IC_{50} values for CYP enzymes were determined in biochemical assays using either human hepatocyte microsomes (2C9, 2C19, 3A4) or recombinant enzymes (11B1, 11B2, 17, 19) with each substrate at its measured K_m value. Substrates were: diclofenac (2C9), omeprazole (2C19), testosterone (3A4), deoxycortisol (11B1), deoxycorticosteroid (11B2), 17a-hydroxypregnenolone (17 lyase), pregnenolone (17 hydroxylase), and testosterone (19). Reactions were analyzed for product using HPLC/MS/MS

methods and IC_{50} values (in µM) were determined by fitting a 4-parameter logistical fit to the dose response data (OpAns, LLC).

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22. The x-ray structure of VT-1598 in complex with *Aspergillus fumigatus*-CYP51 can be found at www.rcsb.org under accession code 5FRB.
23. For the PK study in male Swiss albino mice, VT-1598 was dosed by oral gavage at 5 mg/kg in 20% cremophor EL (in water) (N=3 mice). Time points collected were pre-dose, 0.5, 1, 2, 4, 8, 24, and 48 hours. Plasma levels were measured by HPLC/MS/MS methods and pharmacokinetic parameters were generated by using Phoenix® WinNonLin® Version 6.4.
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