Crystal structures of the fungal pathogen *Aspergillus fumigatus* protein farnesyltransferase complexed with substrates and inhibitors reveal features for antifungal drug design

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Abstract: Species of the fungal genus *Aspergillus* are significant human and agricultural pathogens that are often refractory to existing antifungal treatments. Protein farnesyltransferase (FTase), a critical enzyme in eukaryotes, is an attractive potential target for antifungal drug discovery. We report high-resolution structures of *A. fumigatus* FTase (AfFTase) in complex with substrates and inhibitors. Comparison of structures with farnesyldiphosphate (FPP) bound in the absence or presence of peptide substrate, corresponding to successive steps in ordered substrate binding, revealed that the second substrate-binding step is accompanied by motions of a loop in the catalytic site. Re-examination of other FTase structures showed that this motion is conserved. The substrate- and product-binding clefts in the AfFTase active site are wider than in human FTase (hFTase). Widening is a consequence of small shifts in the α -helices that comprise the majority of the FTase structure, which in turn arise from sequence variation in the hydrophobic core of the protein. These structural effects are key features that distinguish fungal FTases from hFTase. Their variation results in differences in steady-state enzyme kinetics and inhibitor interactions and presents opportunities for developing selective anti-fungal drugs by exploiting size differences in the active sites. We illustrate the latter by comparing the interaction of ED5 and Tipifarnib with hFTase and AfFTase. In AfFTase, the wider groove enables ED5 to bind in the presence of FPP, whereas in hFTase it binds only in the absence of substrate. Tipifarnib binds similarly to both enzymes but makes less extensive contacts in AfFTase with consequently weaker binding.

Keywords: prenyltransferase; posttranslational modification; lipids; isoprenoids; antifungal drugs; crystal structure; enzyme kinetics; farnesyltransferase inhibitors; pathogens; FTI; structure-based drug design

Abbreviations: AfFTase, *Aspergillus fumigatus* protein farnesyltransferase; CnFTase, *Cryptococcus neoformans* farnesyltransferase; DTT, dithiothreitol; ED, ethylenediamine inhibitor; FPP, farnesyldiphosphate; FTase, protein farnesyltransferase; FTI, farnesyltransferase inhibitor; GGase I, protein geranylgeranyltransferase I; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); hFTase, human farnesyltransferase; RT-PCR, real-time polymerase chain reaction; ScFTase, *Saccharomyces cerevisiae* farnesyltransferase

Additional Supporting Information may be found in the online version of this article.

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Figure 1. Chemical structures of protein farnesylation substrates, analogs and products. (A) The reaction catalyzed by protein farnesyltransferase on protein substrates bearing the C-terminal CaaX motif. (B) Structure of the isoprenoid analog, farnesyl diphosphate inhibitor II (FPT-II).⁴¹

Introduction

There is a dearth of therapeutics to treat fungal infections in humans and plants.^{1–4} Current treatment of fungal infections is limited to polyenes (amphotericin B derivatives), echinocandins (caspo-fungin), antimetabolites (flucytosine), and azoles (itraconazole). Unfortunately, toxicity and resistance often limit the use of these drugs, therefore creating an urgent need for low-cost antifungals.⁵ It has been shown that protein farnesyltransferase (FTase) is essential for growth and virulence in several pathogenic fungi and therefore presents an attractive target for the discovery of new antifungals.^{6–8} FTase catalyzes the attachment of the 15-carbon lipid group, farnesyl diphosphate (FPP), to more than 120 protein substrates bearing the C-terminal Ca₁a₂X motif (C = cysteine; a₁, a₂ = aliphatic residues; X = variable residue) [Fig. 1(A)], which include Ras, Ras homologs, and other small G proteins.^{9–11} This lipidation step is critical for localization to membranes of cell compartments, where such modified proteins function.^{11,12} Human FTase (hFTase) has long been the target for the development of cancer therapeutics, which presents an opportunity for repurposing reagents discovered in these studies, provided that these inhibit fungal and not human enzymes.^{11,13–20} Structural studies of FTases from fungal pathogens have revealed significant differences between the human and fungal enzymes in substrate- and product-binding regions, which can be

exploited for the discovery of species-specific FTase inhibitors.^{20–27} Here we report the structure of FTase from the fungal pathogen *Aspergillus fumigatus* (AfFTase) and show that this enzyme also exhibits structural differences from the human enzyme that are sufficient for species-specific inhibition.

Aspergillus is a pathogenic fungus with significant adverse human health and agricultural impact.²⁸ Aflatoxin is produced by *Aspergilli* and is one of the most potent human carcinogens known.^{29–32} In 2012, a fungal meningitis outbreak caused by contaminated steroid injections occurred in the United States and claimed 63 lives of over 750 cases reported at the time of writing (<http://www.cdc.gov/hai/outbreaks/meningitis-map-lare.html>). The index case in this epidemic was caused by *A. fumigatus*. Aspergillosis caused by *A. fumigatus* is the leading cause of death in patients with acute leukemia and recipients of hematopoietic stem cell transplants.^{33–36} Left untreated, invasive aspergillosis can result in mortality rates as high as 100% in certain patient groups, whereas mortality rates remain >30% in certain high-risk immunocompromised patient populations even after treatment with amphotericin B.³⁷ Other species of *Aspergillus* are opportunistic pathogens of field crops (corn, rice, wheat, cassava, peanuts, sorghum, cotton seed, millet, etc.).²⁸ In developing nations, the use of aflatoxin-contaminated grains in fodder reduces animal productivity, diminishing income and reinforcing conditions that promote poor human health.³⁸

Table I. Crystallographic Data Collection and Refinement Statistics

	Data collection			
	FPP (4MBG)	FPT-II-KCVVM (4L9P)	FPP-ED5 (4LNB)	FPP-Tipifarnib (4LNG)
Resolution, Å (Highest resolution shell)	50.00–1.74 (1.77–1.74)	50.00–1.45 (1.48–1.45)	50.00–1.75 (1.78–1.75)	50.00–1.90 (1.93–1.90)
Space group	P2 ₁	P2 ₁	P2 ₁	P2 ₁
Cell dimensions				
<i>a</i>	63.25	63.24	63.32	63.53
<i>b</i>	90.79	90.34	91.25	90.66
<i>c</i>	83.14	83.01	83.05	83.16
γ	110.87°	111.02°	110.93°	111.09°
<i>R</i> _{sym}	8.8 (49.5)	6.9 (59.1)	11.0 (59.0)	12.9 (43.2)
<i>I</i> / σ <i>I</i>	29.9 (2.7)	36.0 (2.0)	22.6 (1.6)	12.3 (1.9)
Completeness (%)	99.8 (97.1)	99.1 (82.8)	97.2 (75.4)	97.3 (81.0)
Redundancy	10.5 (5.5)	10.4 (5.5)	6.2 (2.4)	5.3 (2.7)
Refinement				
Resolution (Å)	23.96–1.75	22.42–1.45	37.07–1.75	30.23–1.90
No. of reflections	89,162	153,459	85,716	66,205
<i>R</i> _{work} / <i>R</i> _{free}	12.2/16.4	12.5/15.2	15.9/18.4	15.5/18.5
No. of nonhydrogen atoms				
Total	7141	7379	6657	7112
Water	700	769	582	615
B-factors				
Protein	10.2	13.6	20.8	16.5
Zn ²⁺	3.44	5.58	10.68	6.91
Ligands	FPP: 6.95	FPT-II: 10.85 KCVVM: 15.99 (occ = 0.7)	FPP: 17.47 ED5: 36.13	FPP: 8.89 Tipifarnib: 11.14
Root mean square deviations				
Bond lengths (Å)	0.011	0.018	0.003	0.003
Bond angles (°)	1.209	1.776	0.795	0.791
Ramachandran				
Favored (%)	98.03	97.87	98.02	97.87
Allowed (%)	1.97	2.13	1.98	2.13
Outliers (%)	0	0	0	0

Results

Protein expression and structure determination

The open reading frames for the α and β subunits of the AffTase heterodimer were amplified from cDNA and cloned into a dual expression vector, pCDFDuet-1, under control of the T7 promoter with the α subunit having a C-terminal hexa-histidine affinity tag.³⁹ Proteins were produced by heterologous expression in C41 (DE3) cells and purified as described previously; approximately 3.5 mg of purified protein was produced per liter of culture.^{20,40} Purified AffTase crystallized in hanging drops using PEG6000. Complexes for FPP only, the FPP analog, FPT-II [Fig. 1(B)] with the Lys-Cys-Val-Val-Met (KCVVM) peptide substrate, and FPP with inhibitors were prepared by mixing protein and ligands prior to crystallization.⁴¹ The structures were determined to 1.45–1.75 Å resolution by molecular replacement using human FTase as the search model (Table I).

Global movements of secondary structure elements widen active site features

The right-handed super helical AffTase α subunit forms a crescent around the globular β subunit

[Fig. 2(A)]; the active site is located within the domain interface, as is the case for all FTases.^{20–27} No electron density is found for the first 70 residues at the N-terminus of the β subunit. Like other fungal FTases, AffTase contains insertions within α -helices and surface loops of the α and β subunits compared with human FTase [Fig. 2(B)]. Although the structures of AffTase and hFTase align with an RMSD of 1.8 Å [Fig. 2(C)], the accumulated effect of these insertions is to widen the active site. We calculated the volumes of the active sites of the AffTase, hFTase (PDB ID **1TN6**), and *C. neoformans* FTase (CnFTase, PDB ID **3Q75**) ternary complexes with FPT-II and Ca₁a₂X peptides [Table II, Fig. 3(A)], using the Computed Atlas of Surface Topography of Proteins (CASTp) server (2.5 Å probe radius), which identifies pockets and voids on a protein structure.^{42–44} The largest pocket identified in each FTase structure corresponds to the active site funnel [Fig. 3(A,B)]. The enclosed volumes of active site funnels are generally larger in fungal FTases, with the calculated volume of the AffTase funnel (3900 Å³) being double that of hFTase

Figure 2. Comparison of the tertiary structures of farnesyltransferases from *A. fumigatus* (AfFTase) and human (hFTase, PDB ID **1TN6**). (A) The α (green) and β (magenta) subunits of AfFTase with substrates FPT-II, the pentapeptide sequence KCVVM (yellow sticks), and divalent metal Zn^{2+} (pink sphere). (B) Insertions in the AfFTase α and β subunits (red) alter its $\text{C}\alpha$ backbone relative to the hFTase subunits, resulting in global movements of secondary structures that widen the active site. An interactive view is available in the electronic version of the article. (C) Superposition of the tertiary structures of AfFTase (green) and hFTase (gray) showing an RMSD of 1.8-Å calculated over homologous regions.

(1900 Å³, PDB ID **1TN6**).²⁷ The larger active site of AfFTase consequently increases the distance between the isoprenoid/peptide substrates and

the residue side chains forming the wall of the active site funnel [Fig. 3(C), Supporting Information A].

Table II. Volume and Surface Area of Active Site Funnels of Fungal and Human FTases Calculated Using the Computed Atlas of Surface Topography of Proteins (CASTp) Server⁴²

Structure	Volume (Å ³)	Surface area (Å ²)
AfFTase ternary complex (PDB ID 4L9P)	3900	1400
hFTase ternary complex (PDB ID 1TN6)	1900	900
CnFTase ternary complex (PDB ID 3Q75)	2800	1300
AfFTase complex with FPP and tipifarnib (PDB ID 4LNG)	2800	1200
hFTase complex with FPP and tipifarnib (PDB ID 1SA4)	1500	800

A 2.5-Å probe radius was used in the experiments.

Figure 3. Global rearrangements of secondary structures lead to wider active sites in fungal FTases. (A) Schematic representation of the active site funnel and the prenylated product exit groove of protein farnesyltransferase (FTase). The substrates farnesylidiphosphate (FPP, green sticks) from the *A. fumigatus* FTase (AfFTase) binary complex and the Cys-Val-Val-Met peptide (yellow sticks) from the AfFTase ternary complex with the FPP analog, FPT-II, are shown. FPP and FPT-II bind in essentially the same manner to the isoprenoid binding pocket of AfFTase. The superimposed structure of the displaced farnesylated peptide product (thin gray lines) of hFTase (PDB ID **1KZO**) is also shown to highlight the position of the prenylated product exit groove relative to the active site funnel. (B) Surface representation of the active site funnels of protein farnesyltransferase from *A. fumigatus* (AfFTase), *C. neoformans* (CnFTase, PDB ID **3Q75**), and human (hFTase, PDB ID **1TN6**) viewed from the top of the active site funnel. Residues whose atoms form the walls of the active site funnel are shown as sticks and colored green (AfFTase), blue (CnFTase), and pink (hFTase). A black line traces the exit groove tunnel adjacent to the active site funnel. Fungal FTases have wider active site funnels arising from insertions in the α and β subunits relative to hFTase. The FPP analog, FPT-II, and peptide substrates bound in the active site are shown as sticks. An interactive view is available in the electronic version of the article. (C) Widening of the active site funnel causes longer distances between substrate (FPT-II and CVVM peptide in AfFTase, cyan; FPT-II and CNIQ peptide in hFTase (PDB ID **1TN6**), gray; and residue atoms in the active site. Selected residues to illustrate the increase in distances are shown in sticks (cyan, AfFTase; gray, hFTase). Distances between atoms are indicated by dashed lines (cyan, AfFTase; gray, hFTase). The positioning of the α helices in the α and β subunits of hFTase result in a narrower active site funnel. The amino acid(s) preceding the $\text{Ca}_1\text{a}_2\text{X}$ cysteine in both structures have been omitted for clarity.

The prenylated product exit groove in the β subunit exhibits the highest degree of sequence and structural variation among FTases.²⁰ In hFTase, the exit groove serves as a binding site for the displaced farnesylated product prior to its release.^{45,46} Superposition of AfFTase, CnFTase, and hFTase structures shows a wider exit groove in the two fungi (Fig. 4).²⁰ Specifically, a stretch of 5 helical residues (143–147 β , β 3 helix) making up one wall of the exit groove in AfFTase is displaced 2–4 Å at the Ca position relative to the corresponding residues in hFTase (92–96 β).

The active site is highly conserved

The Zn^{2+} ion in the active site is essential for catalysis. The 1.45-Å structure of the ternary complex unambiguously confirms the distorted pentacoordinate geometry formed by the universally conserved residues in the primary coordination sphere [Fig. 5(A)], as observed in other FTase structures.^{20–27} The fully extended isoprenoid and peptide substrates in the ternary complex form extensive van der Waals interactions with each other and adopt conformations that correspond to a state in which the thiolate and phosphate groups are not yet aligned for

Figure 4. The prenylated product exit groove in the β subunit is highly diverged in sequence and structure among FTases. Comparison of the exit grooves of the protein farnesyltransferase ternary complexes of *A. fumigatus* (AfFTase, green), *C. neoformans* (CnFTase, purple PDB ID **3Q75**), and human (hFTase, magenta, PDB ID **1TN6**) show that the product exit groove in AfFTase and CnFTase are wider than hFTase, with the $\beta 3$ helix (143–147 β) of AfFTase displaced 2–4 Å relative to that of hFTase. The bound FPP and displaced farnesylated CVIM peptide (light blue sticks) are modeled using a superimposed structure of the hFTase displaced product complex (PDB ID **1KZO**). Amino acid residues that form the walls of the product exit groove are shown as sticks.

catalysis [Fig. 5(B)]. The residues contacting the substrates are conserved and include D432 β (D352 β in hFTase) which is hypothesized to coordinate a Mg^{2+} ion near the diphosphate moiety during catalysis.⁴⁷

Figure 5. A look into the active site of *A. fumigatus* protein farnesyltransferase (AfFTase). (A) A $2F_o - F_c$ map contoured at 3.5σ and calculated to 1.45-Å resolution shows the distorted pentacoordinate geometry of Zn^{2+} coordination. (B) The AfFTase active site showing bound isoprenoid (FPT-II, light green sticks) and peptide (yellow sticks) substrates. Residues that interact with FPT-II (or FPP) by hydrogen bonding (blue dashes) are shown as sticks. The D423 β residue putatively coordinates a Mg^{2+} ion during the reaction that is required for efficient catalysis. The Ca_{1a_2X} peptide (yellow sticks) binds in a fully extended conformation, anchored by coordination with Zn^{2+} (pink sphere) and hydrogen bonding with Q110 α (green sticks). Residues that form the surface of the Ca_{1a_2X} peptide binding site are also shown (purple sticks).

Peptide-induced conformational changes

Substrate binding is known to be ordered: FPP binds first and forms part of the binding surface for the Ca_{1a_2X} peptide substrate.^{48,49} Comparison of the FPP binary and FPT-II-peptide ternary complexes revealed that peptide binding is accompanied by a 2.5-Å motion of the loop between helices 4 and 5 in the α subunit (residues 106–110), which is located adjacent to the bound peptide [Fig. 6(A)]. Residue K107 α is conserved and interacts with the peptide; the alanine mutation of the corresponding residue K164A α in hFTase leads to ~ 30 -fold decrease in the rate of product turnover.^{20,50} Y109 α and Q110 α are also conserved across species. The side-chains of these three residues move in response to peptide binding: K107 α forms a hydrogen bond with the residue (lysine in this instance) preceding the Ca_{1a_2X} motif; Y109 α forms a van der Waals contact with the isoprenoid chain; Q110 α anchors the peptide in the active site by hydrogen bonding with the C-terminus. Re-examination of other FTase structures revealed similar, but small motions [Fig. 6(B)].^{20–27}

Binding groove widening is consistent with variations in steady-state enzyme kinetics

Kinetic studies of hFTase, *Saccharomyces cerevisiae* FTase (ScFTase), and AfFTase (reported here) using isoprenoid and short peptides show that the two fungal enzymes have higher K_M values for both FPP and known cognate peptide substrates compared to equivalent hFTase substrates (Table III).^{48,49,51–53} Tetrapeptides are efficient FTase and geranylgeranyltransferase I (GGTase I) substrates with comparable affinity and reactivity to longer peptides and full-length proteins.^{54–58} Furthermore, the turnover number (k_{cat}) of the fungal enzymes is higher than

Figure 6. Ligand-induced conformational change in the conserved 4α-5α loop of fungal and mammalian protein farnesyltransferases. (A) In *A. fumigatus* FTase (AfFTase), the loop conformation in the ternary complex (FPT-II- (replaced by FPP from the AfFTase binary complex) and KCVVM peptide-bound, green) enables K107α to form a hydrogen bond with the backbone carbonyl of the lysine residue of the KCVVM peptide. Y109α is positioned away from the carboxylate end of the peptide. In the binary complex loop conformation (FPP bound, magenta), the N atom of K107α is moved away from the original position and is unable to form this hydrogen bonding interaction. The conformation of Y109α in this loop conformation clashes with the carboxylate end of the peptide (red arrow). Sequence alignment shows that K107α, Y109α, and Q110α are conserved in mammalian and fungal FTases. An interactive view is available in the electronic version of the article. (B) Similar but smaller ligand-induced loop motions in mammalian FTase. The conformation is shown for different steps in the reaction cycle (bound isoprenoids were omitted in the representations for clarity): apoenzyme (PDB ID **1FT1**, gray); AfFTase with bound FPP analog, FPT-II, and the DDPTASACNIQ peptide (PDB ID **1TN6**, purple), farnesylated CVLS peptide (PDB ID **2H6F**, blue), FPP, and displaced farnesylated CVIM product (PDB ID **1KZO**, green). The Cα backbone of 4α-5α loop of hFTase has less flexibility than the corresponding loop in AfFTase (~1.2 Å shift from apoenzyme to displaced product complex). Residue K164α appears to undergo minimal shift in position; rotamer flipping is observed for Tyr166α and Q167α upon peptide binding (purple arrows).

hFTase.^{48,51,59} Both of these observations are consistent with weaker substrate affinities.^{60–62} Weaker binding of farnesylated product in the widened exit groove could contribute further to higher k_{cat} values by diminishing product inhibition effects that have been observed in hFTase.⁶³

Binding groove widening is consistent with inhibitor selectivities

A preliminary screen of several FTase inhibitors included compounds based on the ethylenediamine scaffold which are known to bind differentially to hFTase and CnFTase by contacting different parts of

Table III. Comparison of the Steady-State Kinetic Parameters of *A. fumigatus* Protein Farnesyltransferase (AfFTase) with Published Values for Human (hFTase) and *S. cerevisiae* (ScFTase)

Kinetic parameters	AfFTase	hFTase ^{a,b}	ScFTase ^c
$K_{\text{M,FPP}}$ (nM)	760 ± 235	15 ± 2 ^a 3.9 ± 0.3 ^b	420 ± 90
$K_{\text{M, peptide}}$ (nM)	1308 ± 340 (KGCVIM) 1840 ± 1100 (KGCVVM)	130 ± 15 ^a (KKSSTKCVIM) 340 ± 30 ^b (GLPCVVM)	2000 ± 1000 (GCVIA)
k_{cat} (s ⁻¹)	0.12 ± 0.01	0.023 ± 0.002 ^a 0.061 ± 0.004 ^b	2.6 ± 0.1

Known cognate C-terminal peptide substrates were used in the published studies and our work: for hFTase, N-Ras (biotinyl-GLPCVVM) and K-Ras4B (biotinyl-KKSSTKCVIM); for AfFTase, genomic RasA (KGCVIM) and the human N-Ras peptide (which corresponds to the C-terminus of a putative protein substrate in the *A. fumigatus* genomic sequence); for ScFTase, the yeast mating pheromone α-factor (dansyl-GCVIA). The C-terminal Ca_{1a2}X motifs are underlined.

^a Ref. 53

^b Ref. 48

^c Ref. 49

Table IV. IC_{50} Values for Inhibitors Measured Against Farnesyltransferase from *A. fumigatus* (AfFTase) and Human (hFTase)

Inhibitor	IC_{50} AfFTase (nM)	IC_{50} hFTase (nM)
ED5	275 ± 78	4050^a
Tipifarnib	2400 ± 700	$0.9\text{--}7.9^b$

^a Ref. 65

^b Ref. 75

the active site in these enzymes, four well-studied anticancer agents (tipifarnib, lonafarnib, L-778,123, L-744,832), and manumycin A, an inhibitor of CnFTase, revealed a candidate that preferentially inhibits hFTase (Tipifarnib; Table IV) and one that preferentially inhibits AfFTase (ED5; Table IV).^{20,64–75} Ternary FPP complexes of these two inhibitors were crystallized to probe structural features that encode specificity.

Ethylenediamine-scaffold inhibitors consist of four moieties that occupy different parts of the FTase active site (Supporting Information B). In hFTase, ED5 binds at both Ca_1a_2X peptide and FPP sites, coordinates the active site Zn^{2+} , and partially blocks the exit groove [Fig. 7(A)].⁶⁵ Consequently, it blocks binding of both FPP and peptide (bi-substrate inhibition). By contrast, in AfFTase, ED5 can bind in the presence of FPP (mono-substrate inhibition): it blocks the Ca_1a_2X peptide site, coordinates Zn^{2+} , and enters the exit groove. The *p*-benzonitrile moiety that occupies the exit groove in hFTase binds to a cleft adjacent to the mobile Y109 α , thereby propping loop 106–110 into the conformation observed in the binary FPP complex [Figs. 6(A) and 7(A)]. The observed change in inhibition mode is a direct consequence of the differences in active site width: hFTase is too narrow to accommodate FPP and ED5, whereas there is sufficient room for both in AfFTase.

In contrast to ED5, tipifarnib binds to AfFTase in an essentially identical way as in hFTase [Fig. 7(B); Supporting Information B]. The decrease in selectivity of Tipifarnib against AfFTase could be a consequence of weaker contacts between the protein and the inhibitor as a consequence of widening of the active site (of either ternary or Tipifarnib complexes), including the product exit groove where the double quinolone group of Tipifarnib enters (Table II).

Widened grooves present opportunity for design of bulkier inhibitors with antifungal selectivity

Complexes of AfFTase with ED5 and Tipifarnib contain an ethylene glycol molecule in the product exit groove, adjacent to the *N*-Boc-piperidin-4-yl-methyl group of ED5 and the double-ring quinolone group of Tipifarnib (Fig. 8). Another ethylene glycol molecule is found in a small pocket adjacent to Y109 α in the Tipifarnib complex, but not in the ED5 complex,

Figure 7. Binding modes of ethylenediamine-scaffold inhibitor, ED5, and Tipifarnib in *A. fumigatus* and human protein farnesyltransferases. (A) Inhibitor ED5 contains moieties that bind to the peptide binding site, mobile loop (orange), and the product exit groove. In *A. fumigatus* farnesyltransferase (AfFTase), ED5 (cyan) binds in the presence of FPP in the active site. In hFTase, ED5 (gray sticks) is competitive with both isoprenoid and peptide substrates, precluding the binding of FPP. In AfFTase, ED5 is competitive with peptide substrate alone. An interactive view is available in the electronic version of the article. (B) The binding mode of tipifarnib in AfFTase (cyan) is conserved with hFTase (gray). In both AfFTase and hFTase, Tipifarnib binds in the presence of FPP (gray, hFTase; cyan, AfFTase). The weaker affinity of Tipifarnib to AfFTase is due to active site widening, as indicated by distances between atoms of the inhibitor and residues that form the active site funnel. Distances are indicated by colored numbers and dashed lines (gray, hFTase; cyan, AfFTase).

since the pocket is occupied in the latter by the *p*-benzonitrile group of the inhibitor. The presence of ethylene glycol molecules (obtained from the cryoprotectant solution during crystal preparation) in this pocket as well as in the product exit groove suggests that further derivatization of ED5 and Tipifarnib to create bulkier ligands may lead to higher affinity and antifungal selectivity by taking advantage of a widened active site.

Discussion

We obtained X-ray structures of AfFTase complexes with substrates at a maximum resolution of 1.45 Å, offering the most detailed view of an FTase to date. Substrate binding is ordered in FTases with FPP

Figure 8. Ethylene glycol molecules in the *A. fumigatus* protein farnesyltransferase (AfFTase) active site suggest routes for inhibitor optimization. The product exit grooves of AfFTase in complex with ED5 (green) and Tipifarnib (magenta) bind ethylene glycol molecules in similar positions (green in ED5 complex, magenta in Tipifarnib complex). An additional ethylene glycol molecule is found in a small cleft in the AfFTase Tipifarnib complex bordered on one side by Y109 α , which is not found in a similar tunnel in the AfFTase ED5 complex due to occupancy by the *p*-benzonitrile moiety of the inhibitor.

binding first and forming part of the molecular surface that interacts with the subsequently bound peptide substrate.^{48,49} Comparison of high-resolution complexes of FPP bound in the presence and absence of peptide clearly revealed that the second substrate-binding step is accompanied by motions of a catalytic loop and rotations of conserved residue side chains.

The widths of the substrate-binding and product exit grooves are key structural features that distinguish hFTase from AfFTase and other fungal FTases. These grooves are significantly wider in the fungal enzymes compared to hFTase. The residues that form the substrate-binding surface are highly conserved, while there is high sequence divergence in the product exit groove. The widening of these active site clefts is a consequence of small shifts in the α -helices that comprise the majority of the AfFTase structure, which occur as a consequence of sequence variation/insertions in the hydrophobic core of the protein. The differences in steady-state kinetic and inhibitor-binding properties of the human and fungal enzymes are therefore encoded by global structural changes.

The species-specific variations in the widths of the binding grooves result in differences in interactions of the enzymes with substrates and inhibitors and therefore present important opportunities for the development of antifungal drugs. We illustrated the basis of this approach by comparing the interaction of ED5 and Tipifarnib with hFTase and AfFTase. In AfFTase, the wider groove enables ED5 to bind in the presence of FPP, whereas in hFTase it binds only in the absence of substrate. Tipifarnib binds in the same way to both enzymes but makes less extensive contacts with AfFTase and binds more weakly as a consequence.

Our findings not only show how sequences can indirectly drive structural changes in distant, highly conserved regions, but also will aid recent efforts to

discover and design new antimycotic drugs targeting FTase at a time when infectious fungal pathogens are on the rise.

Materials and Methods

Cloning and heterologous expression of *Aspergillus fumigatus* farnesyltransferase

The open reading frames encoding the α subunit of FTase/GGTase and the β subunit of FTase were amplified from *A. fumigatus* cDNA using 5'TGAATAGGATCCAATGGAAGGGAAATACTCGTCCGATCCAGAAATG (α , forward), 5'TACAGTTGCGGCCGCTCAAGCAGAGGCAGATATCTCCGTAGCATG (α , reverse), 5'AGATTCATCATATGCCTGTTATCGCAGCGACTGGGAAAC (β , forward), and 5'TACAGAGGTACCCTACAGGTCGAAAGATTGATTTTCGAACCAGAC (β , reverse). The α -subunit PCR fragment was digested using *Bam*HI (underlined) and *Nde*I (underlined) and subcloned into the pCDFDuet-1 vector in multiple cloning site 1 in frame with the 6xHis tag. The β -subunit fragment was digested using *Nde*I (underlined) and *Kpn*I (underlined) and subcloned into multiple cloning site 2 of the pCDFDuet-1 vector that already contained a correctly inserted α -subunit in multiple cloning site 1. The completed expression construct was sequenced at the Duke University Medical Center DNA Analysis facility.

The AfFTase expression construct was transformed into C41 (DE3) cells for expression. Cultures were grown to an OD of 0.7–1.0, and then induced with IPTG (1 mM final concentration) and the temperature of the incubator was lowered to 18°C. Induction continued overnight (~16 hours). Cells were harvested via centrifugation (3000g for 15 min) and purified using the published procedure used for CnFTase. The protein was then concentrated to 10–15 mg/mL, flash frozen in liquid N₂, and stored at –80°C.

Crystallization and structure determination of AfFTase

Substrate complexes of AfFTase with FPP or with the FPP analog, FPT-II, and the Ca₁a₂X peptide KCVVM were formed by incubating 10 mg/mL of AfFTase with a threefold molar excess of each ligand for 30 min on ice prior to crystallization. The crystals were grown using the hanging drop vapor diffusion method at 17°C; 1 μ L of the protein solution was mixed with 1 μ L of the well solution containing 4–10% PEG6000, 600–800 mM LiCl, and 100 mM HEPES pH 7.5. Crystals grew to maximum dimensions (300 μ M $\times$ 15 μ M $\times$ 15 μ M) in three days. Before cryoprotection, crystals were transferred to a stabilization solution of the mother liquor containing 15% w/v PEG6000. The crystals were then transferred stepwise into cryoprotection solution containing the stabilization solution and 25–35% ethylene glycol and flash frozen in liquid N₂.

Diffraction data were collected at 100 K at the Advanced Photon Source, Beamline 22-BM (Argonne National Laboratory), and at Advanced Light Source, SYBILS beamline 12.3.1. X-ray data reduction and scaling were performed with HKL2000.⁷⁶ The initial structure of the AfFTase ternary complex with FPT-II and KCVVM was determined by molecular replacement using the coordinates of hFTase (Protein Data Bank ID **1TN6**) as the input model. The resulting $F_o - F_c$ map revealed clear and continuous electron density in the active site for FPT-II and the KCVVM peptide at 3 σ , enabling fitting of these ligands into the model. Initial model building was carried out in COOT, and refinement with simulated annealing, B-factor randomization, and coordinate shaking were carried out in PHENIX.^{77,78} Later stages of model building and refinement were also performed in COOT and PHENIX using individual coordinate, anisotropic B-factor, occupancy, and stereochemistry and B-factor weight optimization. The resulting structure contained FPT-II with an occupancy of 1.0 and B-factor of 10 $\text{\AA}^2$. The KCVVM peptide refined with occupancy equal to 0.7 and B factor of 16 $\text{\AA}^2$ in the presence of the dominant conformation of a flexible loop region consisting of residues 106–110 of the α subunit. The minor conformation of this loop has an occupancy of 0.3 (B factor of 9 $\text{\AA}^2$) and occurs when peptide is not bound.

To obtain crystals of inhibitor complexes, AfFTase was incubated for 30 min with a threefold molar excess of FPP followed by the ethylenediamine-scaffold inhibitor, *N*-(*N*-*tert*-Butoxycarbonylpiperidin-4-ylmethyl), *N*-(2-[(4-cyanophenyl)-3-methyl-3H-imidazol-4-ylmethyl]-amino)-ethyl) 2-methylbenzenesulfonamide (ED5), or tipifarnib for 30 minutes on ice.^{65,79} Crystals of these complexes (AfFTase-FPP-ED5 and AfFTase-FPP-tipifarnib) were grown, stabilized, and cryoprotected as described above.

The refined model of the AfFTase-FPT-II-KCVVM complex, with ligands removed in the active

site, was used as a search model in the molecular replacement solution of the FPP and inhibitor complexes. Clear electron densities for FPP, ED5, and tipifarnib were observed in the resulting $F_o - F_c$ maps, allowing the fitting of these ligands into the difference density. The structures of AfFTase-FPP-ED5 and AfFTase-FPP-tipifarnib complexes were refined as described for the substrate complex. All AfFTase structures refined with $R_{\text{work}}/R_{\text{free}}$ values from 12 to 19% with excellent geometry (Table I).

Steady-state kinetics

The AfFTase steady-state kinetic constants were determined in a fluorescence assay using FPP and KGCVVM or KGCVIM peptides: diphosphate release was coupled to the activities of yeast pyrophosphatase (Sigma-Aldrich) and a commercially available phosphate sensor (Invitrogen).⁸⁰ The assay mixture contained 5 mM MgCl₂, 10 μ M ZnCl₂, 1 mM Tris (2-carboxyethyl)phosphine (TCEP), 34 U/mL pyrophosphatase, 5 μ M phosphate sensor in 50 mM, FPP, and peptide in 50 mM Tris-NaCl, pH 7.5. Reaction was initiated by adding 5 nM AfFTase, and fluorescence was recorded for 600 s at 470 nm (λ_{ex} = 430 nm) using a spectrofluorophotometer (SpectraMax M5e Multi-Mode Microplate Reader, Molecular Devices) at 25°C. The initial rate at each substrate concentration was calculated using linear regression and were then fit using GraFit (Erithacus Software) to the Michaelis-Menten with (1) or without (2) substrate inhibition:⁵¹

$$\frac{v}{[E]} = \frac{K_{\text{cat}}[S]}{K_M + [S] + \frac{[S]^2}{K_i}} \quad (1)$$

$$\frac{v}{[E]} = \frac{K_{\text{cat}}[S]}{K_M + [S]} \quad (2)$$

where v is the initial rate, k_{cat} is the catalytic rate constant, $[E]$ is the final enzyme concentration, $[S]$ is the concentration of the varied substrate, K_M and K_i are the Michaelis and inhibition constants, respectively. Rates were converted to mM/s units using a standard curve of the phosphate sensor incubated with ultrapure phosphate standards. Measurements were performed in duplicate.

The stock concentration of isoprenoid (dissolved in Tris-NaCl pH 7.5) was determined by phosphate analysis.⁸¹ The peptide was dissolved in DMSO, which was added at a final ratio of 10% in assay mixtures where the peptide concentration was varied. The stock concentration of AfFTase was determined by Bradford analysis.

Determination of FTI Potency against AfFTase

IC₅₀ values were measured using the fluorescence assay described above.⁸⁰ In the assay mixtures, FPP and peptide concentrations were kept constant at 4 μ M and 1 μ M, respectively. Enzyme at 5 nM final

concentration was used to initiate the reaction. The rate of product formation at each inhibitor concentration was measured from the linear portion of the kinetic trace. Percent activity in the presence of inhibitor was then measured based on the rate at zero inhibitor concentration. The IC_{50} for each FTI was then determined using GraFit by fitting the data to the three-parameter equation:

$$y = \frac{100}{[1 + (\frac{x}{IC_{50}})^s]} \quad (3)$$

where y is the percent activity, x is the log of the inhibitor concentration, and s is a slope factor. Measurements were performed in at least duplicate measurements.

Protein Structure Coordinates

Structures presented in this work are available in the Protein Data Bank with the following accession numbers: PDB ID **4MBG** (AffTase binary complex with FPP), PDB ID **4L9P** (AffTase ternary complex with FPT-II and KCVVM peptide), PDB ID **4LNB** (AffTase complex with FPP and ED5), PDB ID **4LNG** (AffTase complex with FPP and tipifarnib).

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