Structures of Cryptococcus neoformans Protein Farnesyltransferase Reveal Strategies for Developing Inhibitors That Target Fungal Pathogens

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Cryptococcus neoformans is a fungal pathogen that causes life-threatening infections in immunocompromised individuals, including AIDS patients and transplant recipients. Few antifungals can treat C. neoformans infections, and drug resistance is increasing. Protein farnesyltransferase (FTase) catalyzes post-translational lipidation of key signal transduction proteins and is essential in C. neoformans. We present a multidisciplinary study validating C. neoformans FTase (CnFTase) as a drug target, showing that several anticancer FTase inhibitors with disparate scaffolds can inhibit C. neoformans and suggesting structure-based strategies for further optimization of these leads. Structural studies are an essential element for species-specific inhibitor development strategies by revealing similarities and differences between pathogen and host orthologs that can be exploited. We, therefore, present eight crystal structures of CnFTase that define the enzymatic reaction cycle, basis of ligand selection, and structurally divergent regions of the active site. Crystal structures of clinically important anticancer FTase inhibitors in complex with CnFTase reveal opportunities for optimization of selectivity for the fungal enzyme by modifying functional groups that interact with structurally diverse regions. A substrate-induced conformational change in CnFTase is observed as part of the reaction cycle, a feature that is mechanistically distinct from human FTase. Our combined structural and functional studies provide a framework for developing FTase inhibitors to treat invasive fungal infections.

Cryptococcus neoformans is an opportunistic human fungal pathogen that can cause life-threatening respiratory and neurological infections. This fungus has become a major pathogen that can cause life-threatening respiratory and neurological infections. This fungus has become a major pathogen in HIV patients (1) and transplant recipients (2). Globally, almost 1 million cases of cryptococcal meningitis are reported each year, with deaths totaling more than half a million (3). Cryptococcus neoformans is a significant threat to global health, particularly in regions in which the incidence of AIDS is high. C. neoformans now exceeds tuberculosis as the fourth most common imminent cause of death by infectious disease in patients with AIDS in sub-Saharan Africa (3).

Currently, invasive fungal infections are treated by a limited number of antifungal agents: polyenes (amphotericin B-based drugs), echinocandins, antimetabolites (flucytosine), and azoles. Of these, only the polyenes, azoles, and flucytosine have activity against C. neoformans infections (4). Drug toxicity and antifungal resistance often limit the use of these medications (5). There is, therefore, an urgent need to develop new antifungal therapeutics. One successful strategy for developing low cost therapeutics for neglected diseases is to repurpose or iteratively improve existing drugs originally developed for other therapies (6–9). For more than a decade, inhibitors of protein farnesyltransferase (FTIs)2 have been under investigation as cancer chemotherapeutics, with a concomitant development of a significant number of lead compounds and identification of the structural properties that guide FTI design (10). Here we report that FTase, therefore, may also be an attractive new target for the development of low cost antifungal therapeutics targeted against C. neoformans.

FTase is a ubiquitous eukaryotic enzyme that catalyzes post-translational lipidation of the C terminus of more than 60 important signaling proteins (10, 11). Protein substrates of FTase bear a C-terminal sequence CAAX motif: cysteine (C), two generally aliphatic residues (AA), and a variable (X) residue. The lipid substrate of FTase is the 15-carbon isoprenoid farnesyl pyrophosphate (FPP). FTase is a zinc-dependent metalloenzyme.

Reaction intermediates have been defined structurally in hFTase by a series of crystallographic snapshots taken along the reaction coordinate and corroborated by kinetic studies (12–15). Throughout the reaction pathway, the enzyme structure remains rigid, whereas the isoprenoid substrate undergoes conformational rearrangement (12, 13, 16). The slow step in the reaction is product release, which is accelerated by the addition of the next lipid substrate molecule (15). A structural interme-

2 The abbreviations used are: FTI, protein farnesyltransferase inhibitor; FTase, farnesyltransferase; hFTase, human FTase; CnFTase, C. neoformans FTase; FPP, farnesylpyrophosphate; NBD-GPP, 3,7-dimethyl-8-(7-nitro-benzoxadiazol-4-ylamino)-octa-2,6-diene-1-pyrophosphate; FSPP, farnesyl S-thiolodiphosphate; MIC, minimal inhibitory concentration; FPII, farnesyl-protein transferase inhibitor II; N-NTA, nickel-nitrito-triacetic acid; CAPSO, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid.
diate of the human enzyme reveals that before its release, the lipitated peptide product is displaced into a shallow, hydrophobic “exit groove” positioned adjacent to the active site (12).

In mammals, FTase is dispensable for adult homeostasis, although it is required during stages of embryonic development (17). In Saccharomyces cerevisiae, deletion of FTase results in alterations of cell fitness, but the enzyme itself is not essential (18). In these organisms, loss of FTase activity can be compensated for by the related enzyme geranylgeranyltrans-ferase-I (19, 20). By contrast, the C. neoformans FTase (CnFTase) is essential for viability (21).

Here we show that several previously developed antican cer FTIs inhibit CnFTase and exhibit C. neoformans fungicial activity. Phase III clinical candidate tipifarnib (22, 23) (R115777, or Zarnestra) and a recently described ethylendiamine-scaffold inhibitor (24–26) demonstrate significant growth inhibition effects on C. neoformans. Manumycin A, a natural product FTI (27), is the most potent FTI tested and exhibits fungicial activity. Treatment with manumycin A interferes with localization of Ras1 at the cell membrane of C. neoformans.

We report the x-ray structures of CnFTase that define its reaction cycle and inhibitor binding modes and identify regions that are structurally divergent relative to hFTase. A substrate-induced conformational change further mechanistically distinguishes CnFTase from hFTase along the reaction pathway. Taken together, these data provide compelling evidence that the essential FTase of C. neoformans is an attractive drug target. Additionally, these studies offer a paradigm by which known FTI scaffolds may be re-purposed to accelerate and lower the cost of novel antifungal inhibitor development.

**EXPERIMENTAL PROCEDURES**

**Antifungal Compound Testing**—Six FTIs were tested for antifungal activity: L-778,123 (28), L-744,832 (29), manumycin A (27), tipifarnib (23), and two ethylenediamine-scaffold inhibitors (24–26) demonstrate significant growth inhibition effects on C. neoformans. Manumycin A, a natural product FTI (27), is the most potent FTI tested and exhibits fungicial activity. Treatment with manumycin A interferes with localization of Ras1 at the cell membrane of C. neoformans.

**Structure and Inhibition of C. neoformans FTase**

**Antifungal Compound Testing**—Six FTIs were tested for antifungal activity: L-778,123 (28), L-744,832 (29), manumycin A (27), tipifarnib (23), and two ethylenediamine-scaffold inhibitors (24–26). Inhibitor stocks were 20 mM in DMSO. Amphoter cin B was used as a positive control (30); its stock concentration was 5.4 mM in DMSO.

Determination of minimal inhibitor concentration (MIC) was performed according to the Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards standard assay, with modification of the growth medium made according to published protocols due to poor growth of C. neoformans in RPMI medium (21, 31, 32). Serial 2-fold dilutions of the drugs tested for MIC were diluted in YNB medium (6.7 g of YNB/liter, 2% dextrose) and added to a C. neoformans cell suspension containing 1 × 10⁶ cells/ml in a 96-well tissue-culture plate (200 μl/well). The plates were incubated in a humidified air incubator at 37 °C, and cell growth was analyzed at 72 h.

Disc diffusion assays were performed by plating fungal cells (2 × 10⁶) on YNB medium (6.7 g of YNB/liter, 2% dextrose, 2% Bacto-agar). Sterile cotton discs were placed on the surface of the agar, and 10 μl of inhibitor stock were placed on the cotton discs. DMSO alone demonstrated no antifungal activity in this assay at these small volumes. The plates were incubated in air at various temperatures for 48–72 h. The diameter of the zone of growth inhibition surrounding the drug-containing disc correlates with the degree of antifungal activity.

**GFP-Ras1 Localization**—To examine the localization of Ras1 in the presence of manumycin A, a C. neoformans strain expressing GFP-tagged RAS1 under control of the endogenous RAS1 promoter (strain CBN157) was constructed using standard molecular biology techniques (33). Briefly, a GFP-Ras1 fusion was integrated into a plasmid containing the endogenous promoter for Ras1, and this construct was integrated into the genome of C. neoformans by biolistic transformation (34). GFP-Ras1 localization was confirmed by epifluorescent microscopy using a Zeiss Axio Imager.A1 fluorescent microscope equipped with a GFP filter set. Images were obtained with a Zeiss AxioCam MRM digital camera.

Strain CBN157 was incubated overnight in YNB medium and diluted 5-fold into fresh YNB medium containing 10-fold serial dilutions of the drug manumycin A (2.5 μM to 250 μM). DMSO without drug was included as a control. After incubation (with shaking) for 4 h at 30 °C, GFP-Ras1 localization in each sample was determined by epifluorescent microscopy.

**Cloning and Heterologous Expression of CnFTase**—C. neoformans cDNA from strain H99, a clinical isolate, was generated as described and used as a PCR template to amplify the genes for the α and β subunit of CnFTase (35). Standard PCR methods were used to amplify the genes with Platinum Taq Hi-Fidelity DNA Polymerase (Invitrogen). The α subunit was amplified using a forward primer with the sequence (the BglII restriction site is underlined) 5’-CTCAAGTAGATCTAATGG-TAACATCGACCTACATCCCTGTATTCA-3’ and a reverse primer with the sequence (the NotI restriction site is underlined) 5’-TACATCGTTGGCGCCGTATTCTTCAGC-3’ and a reverse primer with the sequence (the NotI restriction site is underlined) 5’-TACATCGTTGGCGCCGTATTCTTCAGC-3’. The PCR-amplified gene was subcloned into multiple cloning site I in the pCDFDuet-1 vector (Novagen) in-frame with the Hisα tag and linker. The β subunit was amplified using a forward primer with the sequence (the NdeI restriction site is underlined) 5’-ATTAGGCTCATATGGCGACCAGAATCTCCTTCACTC-3’ and a reverse primer with the sequence (the AvrII restriction site is underlined) 5’-TACGATTACCTGTTGACTTCTTCATCGTTGCGCCGTATTCTTCAGC-3’. The PCR-amplified gene was subcloned into the multiple cloning site II in the pCDFDuet-1 vector. The completed vector was transformed into the BL21 (DE3) Codon Plus-RIL strain of Escherichia coli. Cultures were grown at 37 °C until A₆₀₀ reached 0.8, and the temperature was reduced to 18 °C. Isopropyl 1-thio-β-D-galactopyranoside was added to 1 mM (final concentration), and induction of expression continued for 16 h.

**Purification of CnFTase**—Cell paste was resuspended in a 10-fold volume of Ni-NTA Buffer A (1× PBS, pH 7.4, 300 mM NaCl, 20 mM imidazole) and lysed in a pressurized homogenizer (Microfluidics Corp.). Lysate was clarified and applied to a Ni-NTA-agarose column. The protein was eluted in one step with Ni-NTA Buffer B (Ni-NTA Buffer A + 250 mM imidazole). The Ni-NTA elution was diluted 5-fold into fresh YNB medium containing 10-fold volume of Ni-NTA Buffer A (1× PBS, pH 7.4, 300 mM NaCl, 20 mM imidazole) and applied to a phenyl-Sepharose Column (Amersham Biosciences). A gradient from Phenyl Buffer A to Phenyl Buffer B (20 mM HEPES, pH 7.5, 5 μM ZnCl₂, 5 mM DTT) was used to elute
the protein. Fractions containing CnFTase were identified using SDS-PAGE, pooled, and concentrated using a centrifugal concentrator (Amicon, 50-kDa molecular mass cutoff) to a volume of 10 ml before diluting with Q Buffer A (20 mM HEPES, pH 7.5, 5 μM ZnCl₂, 5 mM EDTA) to a conductivity of 10 millisiemens/cm. The solution was applied to a Q-Sepharose column (Amersham Biosciences) and eluted with a gradient from Q Buffer A to Q Buffer B (20 mM HEPES, pH 7.5, 1 mM NaCl, 5 μM ZnCl₂, 5 mM EDTA). The fractions containing CnFTase were identified using SDS-PAGE and pooled. The pooled protein was concentrated buffer exchanged into its final buffer (Q Buffer B to Q Buffer C). The protein was flash-frozen in liquid nitrogen and stored at −80 °C before crystallization.

Crystallization and Structure Determination—The initial ternary complex of CnFTase with FPP analog FPT-II (36) and CAAX peptide TKCVVM was achieved by incubating the enzyme (10−15 mg/ml) with a 3-fold molar excess of each ligand for 30−60 min on ice before setting up the crystallization drop. Fresh tris[2-carboxyethyl] phosphate (TCEP) from a 0.5 m aqueous stock was added to the protein-ligand solution to a final concentration of 5 mM. Crystals of CnFTase were grown using the hanging drop vapor diffusion method at 17 °C. 1 l of well solution that contained 9−13% w/v PEG 4000, 200 mM NaCl, 50−150 mM Li₂SO₄, and 100 mM 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO), pH 9.5. Crystals appeared in 2−5 days with typical dimensions of 400 × 150 × 150 μm. Before cryoprotection, crystals were transferred to a stabilization solution (mother liquor but with 15% w/v PEG 4000). The crystals were then transferred stepwise into a cryoprotection solution (stabilization solution plus 18% w/v sucrose or ethylene glycol) and flash-frozen in liquid nitrogen.

X-ray diffraction data were collected 100 Kelvin at the Advanced Photon Source beamline 22-ID (Argonne National Laboratory). Data reduction and scaling were performed with HKL-2000 (37) or XDS (38). The initial CnFTase-FPT-II-TKCVVM structure was determined using molecular replacement as implemented by PHASER (39). Although an interpretable electron density maps could be obtained using the coordinates of hFTase as a probe molecule for PHASER, better Z-scores and higher quality initial electron density maps were obtained when a homology model of CnFTase was used as a probe. The homology models of the α and β subunits were generated using the PHyre server (40), with the coordinates of hFTase (Protein Data Bank code 1TN6) as the input model. Initial rounds of model building were carried out using COOT (41) and simulated annealing refinement in PHENIX (42). Later stages of building and refinement were carried out with COOT and REFMAC5 (43) or PHENIX using TLS and individual isotropic temperature factors, with no simulated annealing.

The initial model of CnFTase was built using diffraction data collected on a crystal of the complex of CnFTase with FPT-II (an FPP analog) and the peptide (TKCVVM). Although omitted from the molecular replacement probe, clear electron density for active site ligands at the 3σ level in the Fσ − F structure permits fitting of both FPT-II and the peptide substrate in the active site. The x-ray diffraction data collection and refinement statistics are summarized in Table 3.

Crystallographic Analysis of Additional Substrate and Ligand Complexes—To prepare complexes of substrate analogs and inhibitors, CnFTase was incubated on ice with 3-fold molar excesses of ligand before crystallization drop setup. Mother liquor components were the same for each complex. We were not able to produce CnFTase co-crystals with manumycin A and therefore, used soaking to prepare this complex. Preformed apoCnFTase crystals were transferred to a stabilization solution (mother liquor plus additional 5% w/v PEG 4000) supplemented with manumycin A for a period of up to a week before cryoprotection and x-ray data collection. Concentrations of manumycin A from 10 mM to 100 mM were explored, and in each case, the crystals exhibited a bright yellow color characteristic of the compound.

In the case of the FSPP and DDPTASACNIQ co-crystallization experiment, the ligands were introduced and the crystals grown as described above. Fourier synthesis was used to determine the structure of this complex using coordinates of the FPT-II and TKCVVM ternary complex structure with ligands removed from the active site. Difference electron density maps (Fα − Fc) suggested a mixture of species in the active site; that is, a major species representing unreacted ternary complex (FSPP plus DDPTASACNIQ) and a minor species representing isoprenylated product. The peptide substrate and FSP were fit into the electron density using COOT, and the coordinates were refined in PHENIX. The ligand occupancies were refined in PHENIX, and the final refined model suggests that the FSP-CAAX species was present at >50% occupancy in the crystal.

Crystals of the FSP-CAAX ternary substrate complex were soaked in stabilizing solution (see above) supplemented with 50 mM Mg²⁺ or Mn²⁺ at 17 °C for 2 h before cryoprotection and x-ray data collection. In the case of the divalent ion soaks, only isoprenylated product was observed in the active site, suggesting the divalent ions accelerated the reaction chemistry in the crystal.

To determine the structures of additional ligand complexes, we used Fourier synthesis methods with the coordinates of the α/β heterodimer from which the FPT-II and TKCVVM ligands were removed. In all cases, the ligands could be readily identified in the Fα − Fc difference Fourier map at a level of 3σ. For all complexes but that with manumycin A, the electron density permitted unambiguous fitting of the ligands. In the case of manumycin A, none of the soaking conditions produced crystals for which difference Fourier electron density was unambig-
uous for ligand fitting. Building and refinement of all ligand complex structures were carried out using COOT and REFMAC5 or PHENIX.

**Structure and Inhibition of C. neoformans FTase**

**Results**

**Inhibition of Fungal Cell Growth by Farnesyltransferase Inhibitors—**C. neoformans ras1Δ mutants exhibit a temperature-sensitive phenotype and are unable to grow at 37 °C; additionally, signal transduction through CrnRas1 requires membrane localization via lipidation (33). Here we show that the ras1Δ phenotype can be mimicked chemically by inhibition of FTase in the wild-type strain. C. neoformans was incubated in the presence of various hFTase inhibitors to assess the effect of these compounds on fungal growth (Fig. 1, A and B). Disc diffusion assays, in which the microbial cells are exposed to a gradient of compound surrounding drug-impregnated cotton discs, revealed notable growth inhibition by manumycin A, an ethylenediamine-scaffold inhibitor, and the preclinical antican- 

cer drug tipifarnib (Fig. 1, A and B).

The MICs in liquid media for the inhibitors tested are summarized in Table 1. The MIC of manumycin A compares favorably with clinical antifungal drug amphotericin B, whereas the other FTIs that were tested exhibit significantly higher MIC values. We hypothesized that the decreased efficacy of some compounds may be due to a lack of cellular penetration. Therefore, to address potential confounding effects of the surface polysaccharide capsule on drug entry into the fungal cell, an acapsular mutant strain (47) was also tested (Fig. 1B, Table 1). With this strain, we observe significantly increased antifungal activity by several FTIs, particularly tipifarnib. A potent peptidomimetic inhibitor of hFTase, L-744,832, shows a modest but detectable antifungal effect only in the acapsular strain (Fig. 1B, Table 1). Taken together, these data suggest that optimizing fungal cell delivery might further enhance the effect of these compounds.

To assess whether farnesyltransferase inhibition results in fungal cell killing or merely growth inhibition, we incubated C. neoformans cells to mid-logarithmic phase and exposed them to varying concentrations of manumycin A, the most potent of the currently tested farnesyltransferase inhibitors. Drug concentrations tested were chosen based on the MIC data (Table 1). The viability of the fungal cells over time was assessed by serial quantitative culture. This assay demonstrated rapid cell killing of C. neoformans after 4 h of incubation, suggesting fungicidal activity of manumycin A against C. neoformans at the concentrations tested (Fig. 2B).

**FTase Inhibitor Affects CrnRas1 Localization in Vivo—**To assess the effect of manumycin A on C. neoformans Ras protein localization, a strain expressing an N-terminal-tagged GFP-CrnRas1 fusion protein was incubated in the presence of the

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**Table 2**

**Substrate modification assay examines CAAX selectivity**

The 19 CrnRas-CVVX variants are listed according to X residue identity in the left-most column. To be considered a substrate, a fluorescent band indicating farnesylation of CrnRas was detected in at least one of the CrnRas-CVVX substrate concentrations (between 0.5 and 10 μM) using constant enzyme and lipid concentrations of 1 and 10 μM, respectively. Selectivity was noted when the intensity of the bands differed at equivalent CrnRas-CVVX concentrations between CnFTase and rat FTase. Rat FTase is 97% identical to human FTase overall and a 100% identical active site region. The far right column indicates the percentage of all C. neoformans open reading frames bearing a C-terminal CAAX sequence with the indicated X residue. Approximately 170 ORFs were returned using a Prosite motif scan of the Uniprot database. Sequences were not pruned from this analysis by annotation of whether a mammalian homolog is known to be prenylated (or not). N/A, not applicable.

| X Residue | Rat FTase activity | C. neoformans FTase activity | Selectivity | Frequency of occurrence of C. neoformans CAAX motifs (percentage) |
|-----------|--------------------|-----------------------------|-------------|--------------------------------------------------|
| Leu       | −                  | +                           | CnFTase     | 19.6                                             |
| Ile       | +                  | +                           | None        | 16.2                                             |
| Ala       | +                  | +                           | CnFTase     | 15.5                                             |
| Met       | +                  | +                           | None        | 14.9                                             |
| Val       | +                  | +                           | None        | 10.8                                             |
| Gln       | +                  | +                           | None        | 6.7                                              |
| Asn       | +                  | +                           | None        | 6.1                                              |
| Gly       | −                  | −                           | N/A         | 2.7                                              |
| Tyr       | +                  | +                           | CnFTase     | 2.7                                              |
| Cys       | +                  | +                           | Rat FTase   | 2.0                                              |
| His       | +                  | −                           | Rat FTase   | 2.0                                              |
| Thr       | −                  | +                           | Rat FTase   | 2.0                                              |
| Pro       | −                  | −                           | N/A         | 0.7                                              |
| Ser       | +                  | +                           | Rat FTase   | 0.7                                              |
| Phe       | +                  | +                           | Rat FTase   | 0                                                |
drug and studied by epifluorescent microscopy. This fusion protein was previously demonstrated to be functional, complementing mutant phenotypes of a C. neoformans ras1 mutant strain (33). Incubation of the GFP-CnRas1 strain in varying concentrations of manumycin A resulted in a dose-dependent defect in CnRas1 localization to membranes, consistent with defective farnesylation of this protein (Fig. 2 A). Moreover, the effect of the farnesyltransferase inhibitors was enhanced at 37 °C compared with lower temperatures (data not shown), consistent with the known thermotolerance defect of C. neoformans ras1 mutant strain (33, 48). The defect in GFP-CnRas1 protein localization was noted at drug concentrations below the MIC, suggesting that the altered fluorescence pattern was not due to cell death.

### Crystal Structure of CnFTase

CnFTase (Fig. 3) is a 96-kDa heterodimer composed of an α (336 residues, 40 kDa) and β subunit (520 residues, 56 kDa). Although CnFTase possesses only 34% sequence identity and 47% sequence homology to the human ortholog, the enzyme overall architectures are similar (Fig. 3 c); the root mean square deviation between hFTase and CnFTase over all aligned Ca atoms is 1.9 Å. The 15 anti-parallel α-helices in the α subunit form a crescent-shaped domain that partially envelops the β subunit. The β subunit contains two additional α helices compared with hFTase that do not perturb the α-α barrel core of the β-subunit (Fig. 3 c). The helices correspond to an insertion into a hFTase loop (residues 378β-381β). Other insertions in CnFTase relative to hFTase...
|                  | Apoenzyme         | FPT-II + TKCVVM ternary complex | FSPP + DDPT ASACNIQ ternary complex | Farnesyl-DDPT ASACNIQ product complex | FPP + L-778,123 | FPP + ethylenediamine inhibitor 1 | FPT-II + ethylenediamine inhibitor 2 | FPT-II + tipifarnib |
|------------------|-------------------|---------------------------------|-------------------------------------|--------------------------------------|----------------|-----------------------------|---------------------------|-------------------|
| **PDB ID**       | 3Q73              | 3Q75                            | 3Q78                                | 3Q79                                 | 3Q7A          | 3Q7F                        | 3SFY                      | 3SEX              |
| **Data collection** | Resolution (Å)    | 50-2.30                        | 50-2.15                             | 50-2.20                              | 50-2.50       | 50-2.00                     | 50-2.20                   | 50-2.10           |
|                  | Space group       | P4₁,2,2                         | P4₁,2,2                             | P4₁,2,2                              | P4₁,2,2       | P4₁,2,2                     | P4₁,2,2                   | P4₁,2,2           |
|                  | c (Å)             | 144.04                          | 141.92                              | 143.02                               | 141.90        | 142.71                      | 143.58                    | 141.16            |
|                  | a = β = γ        | 90°                             | 90°                                 | 90°                                  | 90°           | 90°                         | 90°                       | 90°               |
| **R_{sym}**"     |                   | 8.8 (55.3)                      | 11.2 (50.4)                         | 9.6 (57.0)                           | 8.3 (35.4)    | 9.8 (36.5)                  | 11.0 (49.8)              | 8.5 (71.2)        |
| **Completeness (%)"** |                | 97.2 (97.3)                     | 95.8 (96.6)                         | 100 (100)                            | 96.3 (95.0)   | 98.7 (95.0)                 | 99.1 (94.5)              | 100 (100)         |
| **Redundancy"**  |                   | 3.8 (3.7)                       | 4.3 (4.2)                           | 11.0 (10.5)                          | 7.6 (3.0)     | 3.7 (3.1)                   | 6.6 (4.0)                | 7.0 (6.77)        |
| **Refinement**   | Resolution (Å)    | 33.2-2.30                       | 45.2-2.15                           | 45.2-2.20                            | 48.2-2.50     | 48.2-2.00                   | 45.2-2.00                | 47.2-2.10         |
|                  | No. reflections   | 56.751                          | 70.375                              | 65.270                               | 44.790        | 85.427                      | 60.610                    | 73.122            |
|                  | R_{work}/R_{free} | 19.2/22.0                       | 21.3/23.7                           | 18.3/21.4                            | 21.1/25.8     | 18.6/20.3                   | 19.1/21.2                | 17.8/20.3         |
|                  | No. atoms         | 6.358                           | 6.332                               | 6.280                                | 6.322         | 6.350                       | 6.335                     | 6.323             |
|                  | **Bond factors**  | 120                             | 123                                 | 117                                  | 123           | 173                         | 174                       | 110               |
|                  | Zn²⁺              | 612                             | 474                                 | 344                                  | 383           | 721                         | 488                       | 646               |
|                  | **Active site ligands** |           | 38.25                              | 38                                    | 22           | 18                          | 27                       | 28                |
|                  | **Water**         | 38.25                           | 38.01                               | 38.01                                | 38.01         | 38.01                       | 38.01                     | 38.01             |
|                  | **Root mean square deviations** | 38.25                          | 38.01                               | 38.01                                | 38.01         | 38.01                       | 38.01                     | 38.01             |
|                  | Bond lengths (Å)  | 0.007                           | 0.007                               | 0.003                                | 0.004         | 0.006                       | 0.007                     | 0.011             |
|                  | Bond angles (°)   | 0.723                           | 0.969                               | 0.656                                 | 0.737         | 0.940                       | 0.970                     | 1.280             |
|                  | Ramachandran Plot | 89.7                             | 98.5                                | 98.2                                  | 97.5          | 98.2                        | 98.1                      | 98.0              |
|                  | Favored (%)       | 98.7                             | 98.5                                | 98.2                                  | 97.5          | 98.2                        | 98.1                      | 98.0              |
|                  | Allowed (%)       | 100                              | 100                                 | 100                                   | 100           | 100                         | 100                       | 100               |

"Values in parentheses are for highest resolution shell.

*Includes both active site ligands and buffer components ordered at crystal contacts.*
are located at N and C termini or within surface loops remote from the active site and are largely disordered.

A ternary substrate complex of CnFTase with FPT-II and CAAAX (TKCVVM) peptide reveals that the locations of the substrate binding grooves in the active site are conserved relative to hFTase (Fig. 3b). The lipid and peptide bind side-by-side in extended conformations with extensive van der Waals contact between the ligands. The N terminus of the CAAAX motif is anchored at the catalytic zinc ion, and the C terminus is anchored by a conserved glutamine in the α subunit. A putative product exit groove is located adjacent to the substrate binding sites (Figs. 3b and 7) and may accommodate the displaced iso-

**FIGURE 2. Fungal cell killing and altered GFP-Ras1 localization upon manumycin A treatment.** A, shown are images of *C. neoformans* cells expressing a GFP-tagged Ras1 treated with indicated concentrations of manumycin A (or DMSO control). In DMSO control and low concentration samples, GFP-CnRas1 localizes to plasma membrane of cells and endomembranous structures. At high concentrations of manumycin A, however, most GFP-CnRas1 is not localized to plasma membrane and instead exhibits diffuse localization in cytosol. B, a killing assay shows the rapid decrease in viable *C. neoformans* cells in culture when treated with manumycin A at concentrations of 2.5, 5, and 10 μM.
prenylated product, similar to hFTase. By analogy to similar studies in hFTase (26, 49), this groove may play an important role in inhibitor binding in CnFTase. Significant differences between the protein sequences are observed in the peptide binding site and product exit groove.

**Catalytically Active Crystals Reveal Conserved Mechanism of Farnesylation**—In hFTase, the FPP substrate undergoes a relatively large conformational change in which the C1 carbon moves into the vicinity of the catalytic zinc ion (12, 16). The peptide does not move until after formation of the farnesyl adduct, at which point the lipidated product is partially displaced to the exit groove (12). Bond formation is accelerated by Mg$^{2+}$, which is transiently coordinated by an aspartic acid (Asp-352$\alpha$ in hFTase, conserved in CnFTase as Asp-400$\alpha$) and stabilizes the diphosphate leaving group (50). It has been recently postulated that Mg$^{2+}$ lowers the activation energy of the conformational change required for the FPP movement (51).

We also crystallized the enzyme with a CAAX peptide (DDP-TASACNIQ) and a FPP analog, FSPP (Echelon, Inc.), in which the bridging oxygen between the alkyl chain and the diphosphate group is replaced with sulfur. Crystals were soaked in a stabilization solution in the absence or presence of 50 mM Mg$^{2+}$ or Mn$^{2+}$ for 2 h before cryoprotection and x-ray data collection. In the absence of divalent metals in the crystal, FSPP and the CAAX peptide are slowly reactive. Fo$\sigma$-Fc electron density maps indicate some formation of isoprenylated product; however, ligand occupancy refinement in PHENIX indicates unreacted FSPP is present at 50% occupancy. In the presence of Mg$^{2+}$ or Mn$^{2+}$, the substrates are fully converted into isoprenylated product (Fig. 4). The conformations of the unreacted substrate ligands and isoprenylated product in the active site are similar to the hFTase structures (~0.3Å root mean square deviation superposition).

**Helix 4α-5α Substrate-dependent Conformational Change**—No ligand-dependent conformational changes have been observed crystallographically in human protein prenyltransferase enzymes (12, 23, 26, 28, 36, 49, 52–54). Throughout the reaction cycle and upon binding of inhibitors, these enzymes apparently remain rigid, whereas their substrates exhibit significant rearrangements during chemistry. By contrast, we observe a conformational change in CnFTase upon peptide binding, making it mechanistically distinct from hFTase and human protein geranylgeranyltransferase-I. The peptide complex is similar to the human structure (Fig. 5b) but in the apo and inhibitor complex structures the last turn of helix 4α and...
the loop between helix 4α and 5α move outwards by ~7 Å, as measured by the distance between the Cα position of residue Lys-107α, which is located in the middle of the loop. The rearrangement involves residues 99α–108α. The side chain of Lys-107α makes a hydrogen bond with the main chain of the incoming CAAX substrate (Fig. 5, a and b). In hFTase, this conserved lysine plays an important role in stabilizing peptide binding to the enzyme; mutagenesis of the conserved lysine residue to alanine (Lys-164α in hFTase corresponds to Lys-107α) results in a significant decrease in turnover rate (~30-fold) (16). Finally, although a conformational change in this loop in hFTase cannot be ruled out, crystallographic complexes captured for hFTase (apo enzyme, inhibitor-bound, and substrate-bound) all exhibit identical conformations for the 4α–5α loop.

Structural Determinants of CAAX Substrate Selection—The identity of the α2 and X residues determines Caαα2X (cysteine, two generally aliphatic residues, and X specificity-determining variable residue; X residue, the specificity determining residue of the enzyme; mutagenesis of the conserved lysine residue to alanine (Lys-164α in hFTase corresponds to Lys-107α) results in a significant decrease in turnover rate (~30-fold) (16). Finally, although a conformational change in this loop in hFTase cannot be ruled out, crystallographic complexes captured for hFTase (apo enzyme, inhibitor-bound, and substrate-bound) all exhibit identical conformations for the 4α–5α loop.

Recent studies of hFTase suggest that the α2 and X residues are not recognized independently, and residue identities at either position can modulate selectivity at the other (55). Although our substrate diversity does not explore pairwise-encoded specificity, the observed differences in substrate selection between CnFTase and hFTase at the X-position point to the effect of divergence in their active sites. These differences may be exploited for the design of C. neoforms-selective inhibitors.

Product Exit Groove—The product exit groove is the region of the active site that exhibits the highest degree of structural variation between hFTase and CnFTase. This binding site plays an important role in determining enzyme turnover in human protein prenyltransferases, because the rate-limiting step in the reaction cycle is product release (12, 15). In addition to multiple amino acid substitutions, the exit groove in CnFTase is wider than in hFTase (Fig. 7). Specifically, a stretch of five helical residues making up one wall of the exit groove in CnFTase (80β–84β) is displaced 2–4 Å at the Cα position relative to the corresponding residues in hFTase (92β–96β). The exit groove in CnFTase can accommodate a displaced isoprenylated product bound in a mode similar to that observed for hFTase (Fig. 7); however, the kinetics of product release may be altered relative to the human enzyme.

Structural Studies of Re-purposed Inhibitors—Re-purposing and optimizing existing inhibitor scaffolds are important strategies for generating low cost inhibitors for infectious disease
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FIGURE 5. CAAX-dependent conformational switch of helix 4α-5α loop. a, shown is a comparison of helix 4α-5α loop in hFTase (cyan) and CnFTase (orange). Conformations in CAAX-bound state are similar. The CnFTase Lys-107α (Lys-164α, hFTase) makes a hydrogen bond with the carbonyl oxygen of the residue immediately upstream from the CAAX cysteine. b, shown is superposition of CnFTase helix 4α-5α loop in the CAAX-bound conformation (orange) or the apo/non-CAAX complex conformation (gray). The Ca of Lys-107α moves ~7 Å in this transition.

therapies (8, 9). We, therefore, undertook a structural study of several unique FTase inhibitor scaffolds, including those compounds that demonstrate antifungal activity.

We obtained crystal structures of complexes of four small molecule inhibitors belonging to three distinct classes of FTI scaffolds bound to CnFTase. As described above, tipifarnib and ethylenediamine inhibitor #2 demonstrate significant antifungal activity, whereas ethylenediamine inhibitor #1 and L-778,123 do not.

Tipifarnib binds to CnFTase in the binding site for the peptide substrate in a similar manner to that observed in hFTase (28), forming extensive van der Waals interactions with the FPP lipid chain (Fig. 8, B and D). The double ring quinoline group, which forms the core of this inhibitor scaffold, stacks with Tyr-409β (Fig. 8B). One of the two chlorophenyl rings is stabilized by end-on aromatic interactions with Trp-90β and Trp-94β, with Leu84β further contributing to the hydrophobic environ-

ment. Of particular note is the positioning of this inhibitor relative to the α₂ site. The chloride substituent of one of the chlorophenyl rings is oriented directly toward the expanded and divergent distal binding pocket of the α₂ site (Fig. 8, B and D), which is absent in hFTase, being occluded a tyrosine residue (Fig. 6c). Replacement of the chloride with bulkier substituents could, therefore, result in a more potent and selective inhibitor that cannot bind to hFTase.

The complex of ethylenediamine-scaffold inhibitor with CnFTase is similar to the human enzyme complexes with this series of inhibitors (26), including coordination of the catalytic zinc ion by one of the two N-methylimidazole moieties (Fig. 8, A and D). The α-methylbenzyl moiety is positioned in the aromatic α₂ residue binding site, similar to the chlorophenyl ring of tipifarnib, as described above (Fig. 8, A and D). As in hFTase, the p-bromophenyl moiety enters the putative product exit groove and stacks on Tyr-409β (Fig. 8, A and D). As with tipifarnib, the ethylenediamine scaffold can be further derivatized to explore the differences between hFTase and CnFTase in the α₂ site by modifying with bulkier substituents. Additionally, the bromide substituent positioned in the exit groove may be substituted to fully explore the differences between the two proteins in this region (Fig. 8D).

The two compounds that do not demonstrate antifungal activity (a second ethylenediamine scaffold inhibitor and L-778,123) exhibit similar binding characteristics (supplemental Fig. S2). In particular, aromatic moieties in both the exit groove and α₂ residue binding site can be readily derivatized to improve potency of the inhibitor.

Manumycin A is a natural product FTI that possesses two unsaturated alkyl moieties that resemble the lipid chain of FPP, connected by a cyclohexenone epoxide group (27, 56) (Fig. 1C). The epoxide group is essential for FTase inhibition and can be inactivated by reducing agents (27). The triene moiety at the C4 position of the cyclohexenone epoxide is not required for inhibition (27). We obtained crystals of CnFTase in complex with manumycin A by soaking. After structure determination, we observed continuous $F_o - F_c$ difference electron density at a level of 3σ (or higher) in the isoprenoid binding pocket of the active site (supplemental Fig. S1, E and F). This is consistent with steady state kinetics studies, which reveal that manumycin A is a competitive inhibitor with respect to the isoprenoid substrate (27). The electron density map was consistent with multiple binding modes or inhibitor conformations and, therefore, could not be interpreted unambiguously (supplemental Fig. S1, E and F).

DISCUSSION

It has been challenging to develop novel fungicides for the treatment of C. neoformans infections, which have become a significant threat to global health. FTase presents a promising drug target because it is essential for viability of C. neoformans. Human FTase has been the target of extensive drug development studies for cancer therapeutics, and there is, therefore, a relatively large collection of FTIs, many of which have been tested clinically (10). One strategy for the discovery of novel fungicidal agents is to explore whether these FTIs can be repurposed either directly or through additional rounds of opti-
mization. Comparative structural analysis of CnFTase with hFTase is particularly important to the latter strategy because it can reveal critical differences between the enzymes that can be exploited to design modifications of the lead FTIs. Our analysis of the CnFTase structure complexed with substrates and several FTI leads reveals that, although the chemistry catalyzed by CnFTase and hFTase is conserved, there are critical differences in the substrate and product binding sites that clearly can be exploited for the development of CnFTase-specific FTIs.

We observed three features that distinguish the substrate and product molecular recognition between the two enzymes. First, in the C\text{AAX} binding site, a single residue substitution at the a\text{2} site from tyrosine to asparagine resulted in a deeper cavity in this region. In all crystal structures, this cavity is filled with solvent molecules; however, the crystal structures of CnFTase complexed with two FTI scaffolds suggest straightforward modifications to add bulkier substituents to the inhibitors to penetrate this deeper pocket. Second, the prenylated product exit groove is wider in CnFTase relative to hFTase and varies in depth. Band intensity is representative of CAAX farnesylation with fluorescent isoprenoid substrate, NBD-GPP. The figure shows that CnFTase and rat FTase exhibit similar abilities to robustly farnesylate CnRas1-CVVA and -CVVM. However, only CnFTase was able to farnesylate the CnRas1-CVVL substrate.

FIGURE 6. Structural variation in the binding site for the CAAX substrate. a, shown is a comparison of hFTase (gray) and CnFTase (blue ribbon, orange and red sticks) CAAX binding pockets. CAAX peptide (sequence CVIM) is in yellow and gray sticks for CnFTase and hFTase, respectively. CnFTase residues are colored by region; orange for \(X\) residue binding site and red for \(a_2\) residue binding site. Superposition in \(X\) residue binding site illustrates residue conservation but also highlights deviations in backbone position. In the \(a_2\) residue binding site, substitution of tyrosine (hFTase) with asparagine (N413/\(H9252\) in CnFTase creates a deeper \(a_2\) pocket. b, shown is a representative gel from a substrate modification assay showing activity of CnFTase and rat FTase, which was used as a representative mammalian FTase, against three CnRas1-CVVX substrates. Rat FTase is a suitable substitute for hFTase, as it is 100% identical in the active site region. Band intensity is representative of CAAX farnesylation with fluorescent isoprenoid substrate, NBD-GPP. The figure shows that CnFTase and rat FTase exhibit similar abilities to robustly farnesylate CnRas1-CVVA and -CVVM. However, only CnFTase was able to farnesylate the CnRas1-CVVL substrate.

FIGURE 7. Ligand modeling in the product exit groove. Shown is a model of displaced farnesylated product into the CnFTase exit groove (blue) after superposition with hFTase exit groove (gray). Displaced product can be modeled in CnFTase without steric clashes; however, the shape and residue composition of the CnFTase exit groove are different from hFTase.
amino acid composition. The variations observed in the \( a_2 \) site and exit groove provide opportunities to optimize existing inhibitor scaffolds, consistent with the repurposing strategy we have outlined.

The third distinguishing feature of CnFTase, however, presents an opportunity to pursue a different strategy for inhibiting CnFTase. The unexpected conformational change observed for the 4\( \alpha \)-5\( \alpha \) loop of CnFTase results in a molecular surface in the active site with two distinct states that can be individually exploited for inhibitor design. Because the equilibrium between conformations is apparently CAAX-dependent, inhibitors that are CAAX-competitive can be designed to interact with the helix 4\( \alpha \)-5\( \alpha \) loop conformation observed in the Apo structure. An additional strategy may be to employ an allosteric inhibitor.

### FIGURE 8

**Binding mode of inhibitor scaffolds in CnFTase.**

- **A,** shown is the binding mode of ethylenediamine inhibitor #2 (purple) in CnFTase. This inhibitor coordinates the catalytic Zn\(^{2+}\) ion and extends into the exit groove. An \( \alpha \)-methylbenzyl moiety is positioned in the aromatic \( a_2 \) residue binding pocket adjacent to a water-filled cavity.
- **B,** tipifarnib (cyan) coordinates the catalytic Zn\(^{2+}\) ion and binds competitively with respect to the CAAX peptide. Although it does not occupy the exit groove, the carbonyl moiety of the quinoline core is pointed in the direction of the exit groove and may be derivatized to fully explore this cavity.
- **C,** superposition of inhibitor complexes highlights similarities and differences in their binding modes. Neither inhibitor contacts the CAAX residue binding site (orange residues), and only the ethylenediamine inhibitor makes extensive contacts with the exit groove.
- **D,** shown is a schematic representation of inhibitor interactions in the active site. Both inhibitors make van der Waals contact with FPP and coordinate the catalytic Zn\(^{2+}\) ion. Moieties that interact with the exit groove are indicated by the red oval. Interactions with the binding site for the \( a_2 \) residue of the CAAX peptide binding site are indicated with the red semicircle. Derivatives of the inhibitors at these positions are expected to modulate selectivity between CnFTase and hFTase.
that traps the 4α-5α loop in the retracted state that does not interact with the CAAX peptide (57). Such an inhibitor could be used alone to destabilize CAAX binding or as a potency-enhancing agent in combination with a CAAX-competitive inhibitor.

Our crystallographic data provide comparative structural models between the human FTase enzyme and the FTase from an important opportunistic pathogen. These structural studies are complemented by in vivo studies of FTI function in C. neoformans. We show that FTIs have potent antifungal activity. Moreover, application of the FTI manumycin A disrupts CnRas1 localization in vivo. Modification of lead FTIs guided by structural data to optimize potency must be complemented with chemistries that improve fungal cell entry. Taken together, our structural and functional studies lay a foundation for the development of novel FTIs for the treatment of invasive fungal infections.

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