Genetic and Biochemical Studies Establish That the Fungicidal Effect of a Fully Depeptidized Inhibitor of *Cryptococcus neoformans* Myristoyl-CoA:Protein N-Myristoyltransferase (Nmt) Is Nmt-dependent*

(Received for publication, October 2, 1997, and in revised form, January 7, 1998)

Jennifer K. Lodge‡§, Emily Jackson-Machelski‡, Michelle Higgins‡, Charles A. McWherter¶, James A. Sikorski‡, Balekru Devadas§, and Jeffrey I. Gordon‡

From the ‡Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110, and the §Department of Medicinal and Structural Chemistry, G.D. Searle and Company, St. Louis, Missouri 63198

*This work was supported by National Institutes of Health Grant AI38209 and Monsanto Co. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Dept. of Molecular Biology and Pharmacology, Box 8103, Washington University School of Medicine, 660 South Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-7243; Fax: 314-362-7047; E-mail: jgordon@pharmdec.wustl.edu.

*The abbreviations used are: NMT, gene encoding myristoyl-CoA:protein N-myristoyltransferase; Arf, ADP ribosylation factor; kb, kilobase pair; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

*© 1998 by The American Society for Biochemistry and Molecular Biology, Inc. Print in U.S.A.

 Cryptococcus neoformans is a fungal pathogen that causes chronic meningitis in 10% of patients with AIDS. Genetic and biochemical studies were conducted to determine whether myristoyl-CoA:protein N-myristoyltransferase (Nmt) is a target for development of a new class of fungicidal drugs. A single copy of a conditional lethal C. neoformans NMT allele was introduced into the fungal genome by homologous recombination. The allele (nmt487D) produces temperature-sensitive myristic acid auxotrophy. This phenotype is due, in part, to under-myristoylation of a cellular ADP ribosylation factor (Arf) and can be rescued by forced expression of human Nmt. Two isogenic strains with identical growth kinetics at 35 °C were used to test the biological effects of an Nmt inhibitor. CPA8 contained a single copy of wild type *C. neoformans* NMT, HMC1 contained nmt487D plus 10 copies of human NMT. Since a single copy of nmt487D will not support growth at 35 °C, survival of HMC1 depends upon its human Nmt. ALYASKLS-NH₂, an inhibitor derived from an Arf, was fully depeptidized: p-[2-(methyl-1-imidazol-1-yl)butyl]phenyl-acetyl was used to represent the GLYA tetrapeptide, whereas SKLS was replaced with a chiral tyrosin scaffold. Kinetic studies revealed $K_{i\text{(app)}}$ values of 1.8 ± 1 and 9 ± 2.4 μM for purified fungal and human Nmts, respectively. The minimal inhibitory concentration of the compound was 2-fold lower for CPA8 compared with HMC1. A single dose of 100 μM produced a 5-fold greater inhibition of protein synthesis in CPA8 versus HMC1. The strain specificity of these responses indicates that the fungicidal effect was Nmt-dependent. These two strains may be useful for screening chemical libraries for Nmt-based fungicidal compounds with relatively little activity against the human enzyme.

Cryptococcus neoformans is a haploid yeast that causes systemic infection in immunocompromised humans. The organism has tropism for the central nervous system where it produces chronic meningitis. The incidence of infection in patients with acquired immune deficiency syndrome is ~10% (1). New ways of treating cryptococcal meningitis are needed given the limitations of currently available fungicidal and fungistatic agents (e.g. Refs. 2 and 3). An ideal drug target would be a fungal gene product that is expressed under the conditions of infection and that is essential for the viability of the organism. The metabolic pathway and/or substrate specificities of the target protein should be distinguishable from those of the human host. Finally, it would be desirable if the protein is common to many fungal pathogens and is required for their survival.

Studies of *C. neoformans* pathogenesis have revealed several virulence genes. They encode products involved in capsule formation (CAP59 and CAP64), synthesis of melanin (CNLAC1), and mating (MFα). GPA1 functions as a regulator of each of these functions (4). None of these genes is essential for viability (4–8).

Genetic studies have shown that ADE2 (phosphoribosylaminomimidazole carboxylase) is necessary for the growth of *C. neoformans* in cerebrospinal fluid (9) and that calcineurin is required for survival at 37 °C (10). A number of other *C. neoformans* genes have been isolated, but their necessity for growth and/or infection has either not been evaluated by direct genetic tests or the results of such tests have been ambiguous (e.g. Refs. 11–18). One reason for the paucity of such tests is that targeted gene disruption is limited by several factors in *C. neoformans*. Homologous recombination appears to be inefficient (4–7, 19). There are only a few selectable markers available for conducting such tests (12, 20, 21). Moreover, in the absence of a conditional lethal allele, disruption of an essential gene in this haploid organism will produce death, precluding further analysis of the function of the gene.

The *C. neoformans* gene encoding myristoyl-CoA:protein N-myristoyltransferase (EC 2.1.3.97) (NMT) fulfills many of the criteria for an anti-fungal target. Nmt is a monomeric enzyme that catalyzes the co-translational transfer of myristate, a 14-carbon saturated fatty acid, from CoA to the N-terminal Gly residue of nascent proteins. Cellular N-myristoylproteins have diverse biological functions (22). The enzyme appears to be ubiquitously expressed in eukaryotes, including the two organisms that are the principal causes of systemic fungal infections.
in immunosuppressed humans, C. neoformans and Candida albicans (23, 24). In vitro studies of purified orthogonal Nlmts have shown that their acyl-CoA substrate specificities are highly conserved, whereas their peptide substrate specificities are divergent. These differences in peptide recognition have been exploited to develop species-selective peptidomimetic inhibitors (Refs. 25–29; reviewed in Ref. 30). In addition, genetic tests have established that NMT is essential for the growth and viability of both C. neoformans and C. albicans (19, 31).

NMT represents a unique example of an essential C. neoformans gene where a conditional lethal allele has been generated by homologous recombination. This allele was based on a mutant Saccharomyces cerevisiae NMTI allele (ntm1-451D). nmt1-451D contains a single nucleotide substitution that results in replacement of an absolutely conserved Gly, located 5 residues from the C terminus of the enzyme, with an Asp (32). This substitution reduces the affinity of the enzyme for myristoyl-CoA (33). The analogous mutation in this study reduces the affinity of the enzyme for myristate (33), resulting in replacement of an absolutely conserved Gly, located 5 residues from the C terminus of the enzyme, with an Asp (32).

A strain containing several copies of nmt487D, including one at the endogenous locus, was produced (19). It is a myristic acid auxotroph. Withdrawal of myristate produces rapid growth arrest and death within 4 h (19). Virulence studies using isogenic NMT and nmt487D strains and an immunosuppressed rabbit model of cryptococcal meningitis established that genetic attenuation of Nmt activity allows the host to rid itself of an otherwise fatal infection (19).

In the present study, we generated a strain of C. neoformans with a single copy of nmt487D at the endogenous locus. This strain was used to correlate cellular protein N-myristoylation with growth and viability. Moreover, strains expressing wild type C. neoformans or human Nmt were employed to show that a species-selective, fully depitidized inhibitor of the acyltransferase produces fungicidal effects through an Nmt-dependent mechanism.

**EXPERIMENTAL PROCEDURES**

**Strains and Media—**Strains generated during the course of these studies are described in Table I. Two C. neoformans strains, MO49 (ADE2, NMT, and CMD19 (ADE2, nmt487D) were used in this study (19, 20). The ADE2 strain was generated previously (19, 20). A derivative of MO49 with an unmapped insertion of ADE2 was named CAPS in an earlier report (19) but is now renamed CP8 (Cryptococcus with prototrophy for adenine) to avoid confusion with capsule-deficient mutants (34). The following media were used: YPD (1% yeast extract, 2% peptone, 2% glucose); YPD supplemented with 1% Brij 58 (Sigma) and 500 μM myristate or palmitate (NuChek Prep); YNB (yeast nitrogen base; Bio 101); and RPMI 1640 (with glucamine and without bicarbonate; Life Technologies, Inc.).

**Expression Vectors—**pCN36-23 was employed for forced expression of a C. neoformans ADP-ribosylation factor (Arf) in C. neoformans. The plasmid contains three components: (i) the C. neoformans GAL7 promoter from pGUST11 (35) containing an Nco I site in place of the Nde I site at the initiator Met codon (engineered by PCR using 5′-GGTTCGCGCCGTGGAGAGAAGCAGG-3′ and 5′-GGGCCATGTGCACAGAGGGATTGCCAGC-3′) as primers and pGUST11 as the template DNA; (ii) the open reading frame of the C. neoformans ARF gene (24), obtained by PCR of genomic DNA from strain MO49 (the PCR reaction contained 5′-GGGCCATGTGCACAGAGGGATTGCCAGC-3′ which incorporates an NcoI site at the initiator ATG, and 5′-CTTGGCGCGGGCGGTGCAGAGGTTAATGAG-3′ which incorporates an NdeI site at the 3′ end of the coding sequence); and (iii) an NdeI fragment from pTelHyg (21) that includes sequences for maintenance in Escherichia coli and a hygromycin resistance cassette. The ARF insert in pCN36-23 was sequenced in its entirety to verify that no unanticipated mutations had been introduced by PCR.

**Protein N-Myristoylation in C. neoformans**

The site at the initiator ATG of C. neoformans NMTI (ntm1-451D) was engineered by PCR using 5′-GCGCGGCTGCTGCACAGAGGGATTGCCAGC-3′ and 5′-GGGCCATGTGCACAGAGGGATTGCCAGC-3′ as primers and pGUST11 as the template DNA; (ii) the open reading frame of the C. neoformans ARF gene (24), obtained by PCR of genomic DNA from strain MO49 (the PCR reaction contained 5′-GGGCCATGTGCACAGAGGGATTGCCAGC-3′ which incorporates an NcoI site at the initiator ATG, and 5′-CTTGGCGCGGGCGGTGCAGAGGTTAATGAG-3′ which incorporates an NdeI site at the 3′ end of the coding sequence); and (iii) an NdeI fragment from pTelHyg (21) that includes sequences for maintenance in Escherichia coli and a hygromycin resistance cassette. The ARF insert in pCN36-23 was sequenced in its entirety to verify that no unanticipated mutations had been introduced by PCR.

**Western Blots of C. neoformans Nmt—**Five-milliliter cultures of C. neoformans strains were grown overnight at 24 °C in YPD supplemented 500 μM myristate and 1% Brij 58. The cells were then pelleted by centrifugation at 900 × g for 10 min at room temperature, washed once in PBS, pelleted once more, and then were resuspended in 0.25 ml of a solution containing 2% SDS, 80 mM Tris, pH 6.8, and 2 mM Pefabloc (Boehringer Mannheim). Following addition of 0.5 ml of zirconia/silica beads (0.5 mm diameter, Biospec Products), the cells were vortexed (3 pulses of 1 min, alternating with 1 min of incubation on ice). The resulting lysates were boiled for 5 min and clarified by centrifugation at 12,000 × g for 5 min. Glyceraldehyde (final concentration = 5% v/v), 2-mercaptoethanol (2%), and bromphenol blue (0.002%) were added. Equal amounts of lysate were fractionated by electrophoresis through 10% polyacrylamide gels containing 0.1% SDS (37). The separated proteins were then transferred to Immobilon-P (Millipore) transfer membranes (38). Protein blots were probed with a previously characterized rabbit anti-C. albicans Nmt sera (final dilution = 1:1000-fold in blocking buffer) and then developed using enhanced chemiluminescent detection reagents (Amersham) and visualized using reagents and protocols supplied in the Tropix Western-Light kit.

**Arf Protein Mobility Gel Shift Assay—** Fifty-milliliter cultures of C. neoformans strains were grown overnight in YPD, 500 μM myristate, and 1% Brij 58 at 24 °C to an A595 2.0–3.0. The cultures were then centrifuged as above, washed twice in 25 ml of PBS, and resuspended in 4–6 ml of YPD. A 1-ml aliquot was added to 10 ml of YPD or YPD, 500 μM myristate, and 1% Brij 58. Following a 2-h incubation at 24 or 37 °C, cells were pelleted, washed once in 5 ml of PBS, and resuspended in 0.5 ml of a solution containing 4% SDS and 0.125 M Tris-HCl, pH 6.8. Two volumes of 0.5 mm zirconia/silica beads were added, and cells were disrupted by vortexing as described above. Cleared lysates were prepared and denatured, and the cellular proteins were fractionated on 16%–1-mm thick, 12% polyacrylamide gel containing 0.1% SDS (37). Separated proteins were transferred to Immobilon-P membranes. The protein blots were subsequently incubated with a previously characterized rabbit anti-S. cerevisiae Arf1p sera (I40, a generous gift of R. Kahn, Emory University) diluted 1:50,000 in PBS containing 1% gelatin, 0.2% Tween 20, and 0.1% sodium azide. Antigen-antibody complexes were detected using enhanced chemiluminescent detection reagents and visualized using reagents and protocols supplied in the Tropix Western-Light kit.

**Synthesis of C. neoformans Nmt—** Synthesis of this compound was accomplished according to the scheme shown in Fig. 1 and involved reacting the amine (1) with the 2-methyleneimidoacarboxyl acid (2) in the presence of dicyclohexylcarbodiimide and 1-hydroxybenzotriazole (see Ref. 39 for a description of the seven-step synthesis of compound 1). The resulting product was treated with trifluoroacetic acid at ambient temperature, and the final product (SC-61213) was obtained as a tan amorphous powder from a petroleum ether/methylene chloride/methanol precipitation. The 1H NMR and mass spectral data of SC-61213 were consistent with the structure presented in Fig. 1. A stock solution of 40 mM SC-61213 was prepared in sterile deionized water, and aliquots were stored at −20 °C prior to use.

**Kinetic Studies of the Inhibition of Orthologous Nmts by SC-61213—** The effects of SC-61213 on purified C. neoformans, human, and S. cerevisiae Nmts were defined using a previously described two-step
Protein N-Myristoylation in C. neoformans

The amount of Nmt added to the 110-μl reaction depended upon the species: 50–100 ng of C. neoformans Nmt purified from E. coli (19); 30 ng of a homogeneous preparation of E. coli-derived human Nmt (25); or 3.5 ng of purified recombinant S. cerevisiae Nmt1p (41). The final concentration of [3H]myristoyl-CoA was 0.23 μM. An octapeptide representing the N-terminal sequence of a C. neoformans Arf (GLSYSKLL-NH₂) was used as a substrate peptide (final concentration = 10 μM to 2 μM). [3H]Myristoyl-GLSYSKLL-NH₂ was purified from the reaction mixture by reverse phase high pressure liquid chromatography using a C18 10-μm Bondapak column (dimensions = 3.9 × 300 mm; Waters Corp.) and a linear gradient from H₂O, 0.1% trifluoroacetic acid, 0.05% triethylamine to 100% acetonitrile, 0.1% trifluoroacetic acid. The amount of labeled myristoylpeptide recovered was quantitated with an in-line scintillation counter (42). Peptide Kₘ and Vₘₐₓ values were determined for each Nmt from double-reciprocal plots. The Kₘ value at each inhibitor concentration and the type of inhibition were determined using double-reciprocal plots (43). Kₑ values were calculated from Dixon plots (1/ν versus 1/μ; see Ref. 43). All assays were performed at least twice, each time in triplicate.

Antifungal Susceptibility Assays—The minimal inhibitory concentration of SC-61213 was defined by a broth microdilution anti-fungal test performed according to the protocol described by the National Committee for Clinical Laboratory Standards (NCCLS) tentative standard (44). Brieﬂy, RPMI 1640, buffered to pH 7.0 with MOPS or to pH 5.4 with MES, was introduced together with varying amounts of SC-61213 into each well of a 96-well microtiter plate (Falcon). C. neoformans strains CPA8 and HMC1 were then added to the wells according to the NCCLS S. cerevisiae human Nmt (25); or 3.5 ng of purified recombinant zirconia/silica beads as described above. The resulting lysates were each well of a 96-well microtiter plate (Falcon). SC-61213 was deﬁned by a broth microdilution anti-fungal test performed according to the protocol described by the National Committee of SC-61213 was defined by a broth microdilution anti-fungal test performed according to the protocol described by the National Committee for Clinical Laboratory Standards (NCCLS) tentative standard (44).

Protein Synthesis Assays—To determine the effects of SC-61213 on protein synthesis, cells were grown overnight at 30 °C in YNB, pH 7.0, to an Aₙ₅₀₀ of 0.8. One-milliliter aliquots of the culture were treated with either 3.5 μM cycloheximide or 100 μM SC-61213 for 30 min at 30 °C. [14C]Methionine (100 μCi; 1000 Ci or 37 TBq/mmol) was added to each sample, and the cells were shaken for an additional 30 min at 30 °C. Labeled cells were then harvested by centrifugation, washed once in PBS, and resuspended in 250 μl of 4% SDS, 80 mM Tris, pH 6.8. Cells were disrupted by vortexing three times above in the presence of 0.5 ml of zirconia/silica beads as described above. The resulting lysates were then boiled for 5 min and spun at 12,000 × g for 10 min at room temperature. Triplicate aliquots (10 μl each) of the supernatants were spotted onto Whatman Grade 3 filters. Filters were boiled in 10% trichloroacetic acid, washed, and counted in a scintillation counter. All assays were done in triplicate in two independent experiments.

RESULTS AND DISCUSSION

Generation of Isogenic Strains Containing Single Copies of NMT or nmt487D and Characterization of Their Growth Phenotypes—A purified 9.7-kb HindIII fragment from pCN28-14, containing nmt487D and ADE2 as the selectable marker (Fig. 2A), was used to transform a serotype A strain of C. neoformans with an ade2 allele (MO49). Transformants were initially selected on plates lacking adenine and then screened for myristic acid auxotrophy at 24 and 37 °C. Five percent of the adenine auxotrophs exhibited auxotrophy for the fatty acid at 37 °C. Southern blotting conﬁrmed that all myristic acid auxotrophs had undergone homologous recombination at the NMT locus.

Two-thirds of these auxotrophs had a single copy of nmt487D (e.g., poor strain CMD19 in Fig. 2B). Replacement of the endogenous NMT allele was verified by an allele-specific PCR (Ref. 19; data not shown).

Steady state levels of Nmt were deﬁned in cell lysates prepared from isogenic strains containing 1 copy of wild type NMT (MO49), 1 copy of nmt487D (CMD19), or 10 copies of nmt487D (CMD2). Western blot analysis showed a direct correlation between copy number and cellular levels of the acyltransferase during growth at 24 °C in rich medium (YPD) containing 500 μM myristate (Fig. 2C).

The growth properties of these three isogenic strains were characterized further. In the absence of myristate, the CMD19 strain containing a single copy of nmt487D was unable to grow even at 24 °C (Fig. 3A). In contrast, the isogenic CMD2 strain with 10 copies of nmt487D exhibited only modest growth retardation at 24 °C, although the growth defect worsened progressively as the temperature was raised to 37 °C (Fig. 3A). Addition of 500 μM myristate allowed growth of CMD19 (1 copy) to 30 °C and supported growth of CMD2 (10 copies) to 37 °C. However, in both cases, the rescue by myristate is incomplete when compared with an isogenic NMT strain (Fig. 3A). nmt487D produces a fatty acid auxotrophy that is highly speciﬁc for myristate (C14:0). When laurate (C12:0), palmitate (C16:0), or stearate (C18:0) are added to YPD at concentrations equivalent to C14:0, there is no rescue of growth at 24–37 °C for either nmt487D strain (Fig. 3A and data not shown). The selectivity for C14:0 ﬁts with the observed in vitro acyl-CoA chain length speciﬁcity of puriﬁed C. neoformans Nmt (Fig. 3B).

Brij 58 was included in the medium together with C12:0-C18:0 fatty acids to maintain their solubility in YPD. nmt487D produces sensitivity to this detergent: growth of CMD2 (10 copies of nmt487D) but not MO49 (NMT) is inhibited by 1% Brij 58 (Fig. 3A). We were unable to assess the effects of detergent alone on CMD19 (1 copy of nmt487D) because this strain requires myristate at all temperatures surveyed.

A comparison of the morphologic features of isogenic wild type, CMD19, and CMD2 strains, grown to mid-log phase at 24 °C in YPD containing 500 μM myristate and 1% Brij 58, revealed a second phenotypic characteristic imparted by nmt487D: abberant budding and increased cell size (Fig. 3C). Cells with 1 copy of nmt487D exhibited more severe morphologic abnormalities than cells with 10 copies of the allele.

Levels of Arf N-Myristoylation Correlate with the Growth Phenotype Produced by nmt487D—Earlier [3H]myristate labeling studies of a wild type strain of C. neoformans had shown that during log phase growth, an Arf was the most prominently labeled cellular N-myristylated protein (45). An ARF gene was subsequently recovered from C. neoformans and sequenced (24). Co-expression of C. neoformans Nmt and this Arf in E. coli, a bacterium with no endogenous Nmt activity, conﬁrmed that the Arf is a substrate for the acyltransferase (24). N-Myristoylation of Arfs in E. coli changes their electrophoretic mobility during SDS-polyacrylamide gel electrophoresis: acylation produces more rapid migration compared with the corre-
sponding nonmyristoylated recombinant protein (24, 38). This mobility shift can be used to assay levels of protein N-myristoylation in eukaryotic cells. For example, in *S. cerevisiae* Arf1p and Arf2p are necessary for growth and dependent upon their covalently bound myristoyl moiety for function (46–48). Western blot analysis of cellular lysates prepared from isogenic *S. cerevisiae* strains containing (i) *NMT1* or *nmt1–451D* (corresponding to *C. neoformans* *nmt487D*) and (ii) wild type or null alleles of *ARF1* or *ARF2* established that a $\geq 50\%$ reduction in Arf1p/Arf2p acylation is associated with a loss of viability (38). The gel mobility shift assay was used to define levels of Arf N-myristoylation in isogenic *C. neoformans* strains containing *NMT*, 10 copies of *nmt487D*, and 1 copy of *nmt487D*. Before doing so, an initial series of Western blots were prepared from *E. coli* strains that produced the *C. neoformans* Arf without Nmt (nonmyristoylated Arf standard) or the Arf and *C. neoformans* Nmt (N-myristoylated Arf standard). Based on Coomassie staining of SDS-polyacrylamide gels containing an equivalent amount of total cell proteins from the two bacterial strains, we were able to conclude that the steady state level of each Arf isoform was equivalent in their respective lysates (data not shown). The Western blots were then probed with rabbit polyclonal antibodies to purified *C. albicans* Nmt. These antibodies have been shown previously to cross-react with purified orthologous Nmts (24).

![Image of the diagram](https://example.com/image.png)

**Fig. 2.** Southern and Western blot analysis of *C. neoformans* strains with one or more copies of *nmt487D*. 

A, restriction maps of the cloned *C. neoformans* *NMT* gene (*pCN25-28*) and the targeting vector used to generate *nmt487D* by homologous recombination (*pCN28-14*). The location of the Gly$^{487}$ to Asp mutation in *nmt487D* is indicated by the downward pointing arrow. The sizes of internal *Eco*RI and *Hind*III fragments are noted (H, *Hind*III; E, *Eco*RI). B, Southern blots of genomic DNAs isolated from the parental *NMT, ade2* strain (MO49), an *NMT, ADE2* derivative of MO49 (CPA8), and two strains with *nmt487D* (CMD2, CMD19). C, Western blot containing equivalent amounts of total cellular protein isolated from MO49, CMD2, and CMD19 during log phase growth at 24 °C in YPD/myristate. The blot was probed with rabbit antibodies to purified *C. albicans* Nmt. These antibodies have been shown previously to cross-react with purified orthologous Nmts (24).
to each plate, and the plates were incubated at 24–37 °C for 3 days. Equivalent numbers of cells from the indicated strains were transferred for comparison of isogenic *C. neoformans* (*Ref. 42*). Mean values ± S.D. are plotted.

![Diagram A](image)

**FIG. 3.** Effects of nmt487D on growth and morphology. **A**, comparison of isogenic *C. neoformans* strains containing NMT or nmt487D. Equivalent numbers of cells from the indicated strains were transferred to each plate, and the plates were incubated at 24–37 °C for 3 days. **B**, acyl chain length specificity of purified *C. neoformans* Nmt. Assay conditions are described in Ref. 19 (substrate peptide = GAR[H]ASVLS-NH2; Ref. 42). Mean values ±1 S.D. are plotted. **C**, cellular morphology of CPA8 (NMT), CMD2 (10 copies of nmt487D), and CMD19 (1 copy nmt487D) after a 12-h incubation at 24 °C in liquid YPD, 500 μM myristate, 1% Brij 58. All photographs were taken using Nomarski optics at the same magnification. CMD2 and CMD19 cells appear clumped because of their apparent failure to separate after division (e.g. arrows), although the phenotype appears worse in the CMD19 strain.

Western blots of total cellular proteins were probed with rabbit antibodies raised against purified *S. cerevisiae* Arf1p. The blots show the relative amounts of N-myristoylated and non-myristoylated isoforms (myr-Arf and Arf) in isogenic *C. neoformans* strains grown under four different conditions: + and – indicate the presence or absence of 500 μM myristate, 1% Brij 58 in YPD. The blot shown in the left panel contains a set of *C. neoformans* Arf standards produced in *E. coli* in the presence or absence of *C. neoformans* Nmt. The N-myristoylated Arf standard migrates faster than the non-myristoylated standard.

![Diagram B](image)

**FIG. 4.** Effects of nmt487D on Arf N-myristoylation in vivo. Western blots of total cellular proteins were probed with rabbit antibodies raised against purified *S. cerevisiae* Arf1p. The blots show the relative amounts of N-myristoylated and non-myristoylated isoforms (myr-Arf and Arf) in isogenic *C. neoformans* strains grown under four different conditions: + and – indicate the presence or absence of 500 μM myristate, 1% Brij 58 in YPD. The blot shown in the left panel contains a set of *C. neoformans* Arf standards produced in *E. coli* in the presence or absence of *C. neoformans* Nmt. The N-myristoylated Arf standard migrates faster than the non-myristoylated standard.

that the effect occurs in a gene dose-dependent fashion. CMD19 (single copy of nmt487D) was subsequently transformed with the ARF expression vector. The vector contained a complete *C. neoformans* ARF open reading frame under the control of the galactose-inducible *C. neoformans* GAL7 promoter and a hygromycin resistance gene (HYGB) under the control of a constitutively expressed actin gene promoter. Transformants were selected at 24 °C on YPD supplemented with myristate and hygromycin. Fig. 5 illustrates the growth phenotype of a transformant (AMC1) which, based on Southern blot analysis, has multiple copies of *GAL7-ARF* DNA integrated into its genome. In YP-dextrose plus myristate, there is no rescue of growth at 33 °C. In YP-galactose plus myristate, partial rescue of growth was observed at this temperature. Similar results were obtained with other strains containing multiple copies of *GAL7-ARF*. These findings support the conclusion that this Arf plays a role in defining the growth properties of an nmt487D strain.

**Complementation of nmt487D by Human Nmt—An in vivo test was designed to examine whether human and *C. neoformans* Nmts have distinct substrate specificities for *C. neoformans* proteins.** The test consisted of expressing wild type fungal and human Nmts in an nmt487D recipient strain and defining cellular phenotypes under conditions where the activity of the "endogenous" nmt487D enzyme was insufficient to support growth. CMD19 (one copy of nmt487D) was chosen as the recipient strain for transformation with orthologous Nmts because it has a distinctive, easily scored phenotype (strict myristate requirement) that is stably maintained (reversion frequency <10⁻³). *C. neoformans* NMT was introduced into CMD19, and myristate acid prototrophs were selected at 35 °C on YPD. The efficiency of transformation averaged 210 colonies/μg DNA (n = 10 experiments). Southern blot analysis of 18 randomly selected myristic acid prototrophs revealed that NMT had been integrated into the CMD19 genome in each case, with an average copy number of 3.

The open reading frame of a human Nmt cDNA was placed under the control of a 1.4-kb fragment representing the region 5' to the initiator ATG codon of *C. neoformans* NMT. We assumed that this fragment was sufficient to initiate efficient transcription and translation of human Nmt. A 2.4-kb fragment representing the region 3' to the TAA stop codon of *C. neoformans* NMT was placed downstream of the human Nmt open reading frame. When this human Nmt expression vector was introduced into CMD19, the yield of myristic acid prototrophs was significantly lower than that obtained in parallel experiments with *C. neoformans* NMT DNA (average differ-
ence = 5-fold, n = 9 transformations). Southern blot studies of 19 randomly selected myristic acid prototrophs disclosed a significantly higher average copy number for the human compared to fungal NMT (6 versus 3; p < 0.05).

The growth properties of six strains were then compared in YPD medium. MO49 is the NMT, ade2-containing parent of all of the strains tested. CPA8 was obtained when MO49 was transformed with an ADE2 plasmid (see Table I). CMD19 and CMD2 were generated when MO49 was transformed with nmt487D, ADE2 and, as noted above, have 1 and 10 copies of nmt487D. The other members of the panel were obtained from CMD19 and included CMC2 (2 copies of C. neoformans NMT), CMC1 (10 copies of C. neoformans NMT), HMC2 (2 copies of human NMT), and HMC1 (10 copies of human NMT).

With the exception of CMD19 and CMD2, all strains grew on YPD/agar at 24–37 °C. Growth kinetics were subsequently defined in liquid YPD. No remarkable differences were observed at 24 and 37 °C between the strains containing 1, 2, or 10 copies of C. neoformans NMT (e.g. MO49, CMC2 in Fig. 6A, plus data not shown). The isogenic nmt487D strain complemented with 2 copies of human Nmt DNA showed a slight growth delay at 24 °C and a significant growth defect at 37 °C, analogous to the phenotype of a strain with 10 copies of nmt487D (compare HMC2 and CMD2 in Fig. 6A). Two copies of human NMT only produced partial "correction" of the morphologic abnormalities seen in its CMD19 parent (data not shown). The nmt487D strain with 10 copies of human Nmt DNA grows as well as the strains with wild type C. neoformans NMT (compare HMC1 with MO49 and CMC2 in Fig. 6A) and had normal morphology.

When GLSVSKLL-NH₂, an octapeptide representing the N-terminal sequence of the known C. neoformans Arf, was used as a substrate for purified human Nmt, acylpeptide production was 6.5-fold greater at 37 °C compared with 24 °C (Fig. 6B). When grown in YPD alone, the strain with 2 copies of human NMT (HMC2) produced no improvement in the extent of Arf myristoylation at 24 °C compared with its CMD19 parent (compare Fig. 6C and Fig. 4). However, at 37 °C, human Nmt produced a clear improvement in the extent of Arf myristoylation (50% versus <25%; Fig. 6C and Fig. 4). This finding is consistent with the activity profile of purified human Nmt at 24 and 37 °C.

CMD19 shows impaired growth at 24 °C in YPD. HMC2 shows "normal" growth at 24 °C compared with the wild type MO49 parent, even though the extent of Arf myristoylation in HMC2 is equivalent to that in CMD19. Together, these findings suggest that the extent of N-myristoylation of proteins other than Arf defines the growth properties at 24 °C of strain HMC2. Our results also imply that human and C. neoformans Nmts have overlapping yet distinct substrate specificities in vivo and that C. neoformans produces one or more essential fungal proteins that cannot be adequately acylated by the human Nmt produced in HMC2.

Use of Isogenic Strains Expressing Human or C. neoformans Nmt to Identify an Nmt-dependent Fungicidal Inhibitor of the Enzyme—Isogenic strains of C. neoformans expressing wild-type fungal or human Nmt can be used to establish whether an in vitro inhibitor of the purified acyltransferase with anti-fungal properties exerts its growth inhibitory effect through in vivo inhibition of the enzyme. Specifically, if an Nmt inhibitor is selective for the fungal compared to human enzyme in vitro, and if that compound also produces a greater degree of inhibition of growth of a fungal Nmt-producing, compared to human Nmt-producing strain, then an Nmt-dependent mechanism for its biological effect can be invoked.

In Vitro Characterization of a Fully Depeptidized Inhibitor of C. neoformans and Human Nmts—Nmt has an ordered reaction mechanism: the apoenzyme first binds myristoyl-CoA, forming a binary Nmt:myristoyl-CoA complex; peptide then binds, forming a ternary complex; this is followed by catalysis and release of the CoA and myristoylpeptide products (41, 49–51). As noted in the Introduction, the acyl-CoA substrate specificities of orthologous Nmts are highly conserved (30, 52), whereas their peptide substrate specificities are somewhat different.

We have recently identified potent and selective peptidomimetic inhibitors of C. albicans Nmt (25–27, 29, 30, 52, 53). As with C. neoformans, Arfs are the most prominent C. albicans N-myristoylproteins labeled with [3H]myristate during log phase growth (45). Alanine scanning mutagenesis of an octapeptide derived from the N-terminal sequence of an Arf (GLYASKLS-NH₂) revealed that Gly³, Ser⁶, and Lys⁸ play the most important role in recognition by the peptide binding site of C. albicans Nmt (28). Substitution of Ala for Gly³ yielded an inhibitor (ALYASKLS-NH₂) that was competitive for peptide and noncompetitive for myristoyl-CoA. Replacement of the ALYA tetrapeptide with an 11-aminoindanecanoyl group and replacement of the C-terminal Leu-Ser with N-cyclohexylethyl lysinamide moiety produced a dipeptide inhibitor that was more potent than the starting octapeptide inhibitor (28). Increased potency and selectivity for C. albicans versus human

### Table I

| Strain                | Parental strain | How derived                     | Genotype                          | Ref. |
|-----------------------|-----------------|---------------------------------|-----------------------------------|------|
| MO49                  | H99 (clinical isolate) | Mutagenesis                    | ade2                              | 20   |
| CPA8                  | MO49            | Transformed/pADE2dApa           | ADE2                              | 19   |
| CMD2                  | MO49            | Transformed/pCN28–14            | ADE2, nmt487D (10 copies)         | 19   |
| CMD19                 | MO49            | Transformed/pCN28–14            | ADE2, nmt487D (1 copy)            | This work |
| AMC1                  | CMD19           | Transformed/pCN36–23            | ADE2, nmt487D (1 copy), GAL-ARF    | This work |
| HMC1                  | CMD19           | Transformed/pHS39–57            | ADE2, nmt487D (1 copy), hNMT-ARF   | This work |
| HMC2                  | CMD19           | Transformed/pHS29–57            | ADE2, nmt487D (1 copy), hNMT-ARF   | This work |
| CMC1                  | CMD19           | Transformed/pCN25–28            | ADE2, nmt487D (1 copy), hNMT-ARF   | This work |
| CMC2                  | CMD19           | Transformed/pCN25–28            | ADE2, nmt487D (1 copy), NMT-ARF    | This work |

*Fig. 5. Partial rescue of a nmt487D strain by forced expression of C. neoformans ARF. Growth phenotype of CMD19 without the plasmid and with the ARF plasmid (AMC1). Equal numbers of CMD19 and AMC1 cells were spotted onto media containing either glucose or galactose as the carbon sources (YPD and YP-GAL, respectively), 500 μM myristate and 1% Brij 58. Plates were incubated at 24 or 33 °C for 3 days.*
Nmt was obtained by substituting a 2-methylimidazole for the N-terminal glycin amide and rigidifying the flexible undecanoyl chain with a 4-substituted phenylacetyl group. This compound (SC-58272) is a competitive inhibitor ($K_i = 30 \text{ mM}$ using GNAASARR-NH$_2$ as the peptide substrate) and has 250-fold selectivity for the C. albicans enzyme (27). Unfortunately, it produces no growth inhibition when added to log phase cultures of C. albicans or C. neoformans, even at concentrations up to 100 $\mu$M (Ref. 38 plus data not shown).

The Ser-Lys dipeptide that remains in SC-58272 is a potential site for cleavage by cellular proteases and may contribute to the lack of biological activity in the compound. Therefore, we synthesized a derivative (SC-61213) that lacks any peptide bonds (see Fig. 1).

The $p$-(2-methyl-1-imidazol-1-yl)butylphenylacetyl moiety present in SC-58272 was retained in SC-61213 and used to represent the N-terminal ALYA tetrapeptide of the parental ALYASKLS-NH$_2$ inhibitor. The C-terminal SKLS was represented in SC-61213 by a chiral tyrosinol scaffold. The imidazole group provided a mimic for the essential N-terminal amino recognition element in Ala$^1$ of ALYASKLS-NH$_2$. The critical Ser$^9$ hydroxyl was retained in the form of an alcohol, and the important $\epsilon$-amino group of Lys$^6$ was represented in the form of a 3-(3-aminobutyl) side chain. The hydrophobic interactions provided by Leu$^7$ were provided by the aromatic 4-cyclohexyl-ethyl moiety of SC-61213.

A discontinuous Nmt assay was used to define the effects of SC-61213 on purified C. neoformans Nmt. GLSVSKLL-NH$_2$, representing the N-terminal sequence of C. neoformans Arf, was used as the substrate. In the absence of inhibitor, double-reciprocal plots revealed a $K_m$ for the octapeptide of 0.6 ± 0.2 $\mu$M and a $V_{max}$ of 16,000 ± 1700 pmol myristoylpeptide/min/mg enzyme. Double-reciprocal plot analysis of C. neoformans Nmt activity at different concentrations of SC-61213 suggested a noncompetitive type of inhibition (Fig. 7A). Secondary (Dixon) plots were most consistent with noncompetitive or mixed type inhibition and yielded an apparent $K_i$ of 1.8 ± 1 $\mu$M (Fig. 7B).

GLSVSKLL-NH$_2$ is also a substrate for human Nmt ($K_m = 0.35 ± 0.15 \text{ mM}$, $V_{max} = 4400 ± 700 \text{ pmol/min/mg}$) and therefore was used to assess the effects of SC-61213 on the human enzyme. In contrast to C. neoformans Nmt, double-reciprocal plot analysis suggested an uncompetitive type of inhibition (Fig. 7C). Dixon plots revealed an apparent $K_i$ of 9 ± 2.4 $\mu$M (Fig. 7D). When the octapeptide was used as a substrate for S. cerevisiae Nmt1p ($K_m = 22.5 ± 1.1 \text{ mM}$, $V_{max} = 22,000 ± 2800 \text{ pmol/min/mg}$), a similar uncompetitive type of inhibition was observed ($K'_i = 4.3 ± 1.1 \mu$M; Figs. 7E and 7F).

These studies establish that an octapeptide substrate containing seven peptide bonds and eight chiral centers can be reduced to a nonpeptide inhibitor with one chiral center that is ~5-fold selective for C. neoformans versus human Nmt. The kinetic data suggest that the mode of interaction of the inhibi-

![Fig. 6. Effects of forced expression of human Nmt on growth kinetics and Arf N-myristoylation in a C. neoformans strain containing nmt487D. A, strains were incubated at 24 and 37 °C in YPD: MO49 ( ), single copy C. neoformans NMT), CMD2 ( ), 10 copies C. neoformans NMT); HMC1 ( ), 1 copy nmt487D/10 copies human NMT), and HMC2 ( ), 1 copy nmt487D/2 copies human NMT). In separate experiments, CMC1 (1 copy nmt487D/10 copies C. neoformans NMT) and CPA8 (an ADE2 derivative of MO49 with a single copy of C. neoformans NMT) had growth kinetics indistinguishable from those of MO49, HMC1, and CMC2 (not shown). B, myristoylpeptide production by purified human Nmt at various temperatures. Assay conditions were as follows: $[\text{H}]$myristoyl-CoA = 0.23 $\mu$M, substrate peptide (GLSVSKLL-NH$_2$ from the N-terminal sequence of a C. neoformans Arf) = 1 $\mu$M (three times its apparent $K_m$); human Nmt = 90 ng/ml; incubation time = 10 min. All assays were performed in triplicate on a least two separate occasions. Mean values ± 1 S.D. are plotted. C, assay of Arf N-myristoylation in HMC2 cells.
itor with *C. neoformans* Nmt is different from its interaction with human or *S. cerevisiae* Nmts. Multiple sequence alignments of the six known orthologous Nmts have disclosed that the 416–529 amino acid enzymes contain 105 absolutely conserved resides that are widely distributed throughout the primary structure (33). When compared with the other Nmts, the *C. neoformans* enzyme has a unique 22–39-residue insert in its C-terminal third. The inserted sequence begins with three prolines. It remains to be established whether this insert contributes to the enzyme's apparently distinctive interaction with SC-61213.

Characterization of the Biological Effects of SC-61213—

*CPA8* (*NMT*) and *HMC1* (*nmt487D* plus 10 copies of human *NMT*) were chosen to test whether SC-61213 has any antifungal activity. These isogenic myristic acid prototrophs have similar growth kinetics at 35 °C in standard liquid medium (Fig. 8A) and, based on Western blot analysis, have similar steady state levels of Nmt (data not shown). At this temperature, a single copy of *nmt487D* will not support growth (see above), so growth and survival of HMC1 cells depends upon their human Nmt. SC-61213 is selective for the fungal compared with human enzyme *in vitro*. Therefore, a greater degree of inhibition of CPA8 compared with HMC1 cell growth would indicate an Nmt-dependent mechanism for its biological effects.

We examined the effects of two-fold serial dilutions of the SC-61213 (1.6–100 μM) on growth and survival of the CPA8 and HMC1 strains. A single dose of each concentration of the compound was added to logarithmically growing cells. Cells were then incubated at 35 °C in standard medium at pH 5.4 or 7.0, and the *A*₅₅₀ was defined 48 and 72 h later. SC-61213 inhibited growth in a strain-dependent manner (Fig. 8A). The minimal inhibitory concentration was 2-fold greater for HMC1 compared with CPA8 at pH 5.4 (80 versus 45 μM at 48 h; 82 versus 42 μM at 72 h). This 2-fold difference was observed in two independent experiments, each done in triplicate. At pH 7.0, the difference in minimal inhibitory concentration between strains was less pronounced (e.g. 45 versus 31 μM at 48 h).

To determine whether SC-61213 was fungicidal versus fungistatic, logarithmically growing CPA8 cells (10⁶ colony-forming units/ml) were treated with a single dose of the compound (final concentration = 100 μM in YNB at pH 7.0). The number of colony forming units was defined 24, 48, and 72 h later. After 24 h, no viable cells were detectable (data not shown). The effect could not be simply attributed to cell lysis; loss of viability was not accompanied by a change in *A*₅₉₀ of the culture or a
demonstrable reduction in cell number (defined using a hemocytometer). The fungicidal effect of the single dose of SC-61213 was sustained for 72 h. Untreated control cells continued to grow during this period.

An Arf gel shift assay, performed on CPA8 cell lysates harvested 2 h after administration of SC-61213 (100 μM), failed to detect any non-myristoylated Arf (data not shown). Since a protein de-myristoylation activity has not been found in eu-karyotes (e.g., Ref. 54), production of non-myristoylated Arf should be dependent upon new protein synthesis. It is possible that the failure to detect non-myristoylated Arf in SC-61213-treated CPA8 cells was due to rapid inhibition of translation. To test this hypothesis, logarithmically growing CPA8 cells were treated with 100 μM SC-61213 for 30 min and then pulse-labeled for 30 min with [35S]methionine. A 98% inhibition of protein synthesis was observed (Fig. 5B). The magnitude of this inhibition was similar to that produced by a standard dose of cycloheximide (3.5 μM; see Fig. 5B). A single 100 μM dose of SC-61213 produced 5-fold less attenuation of protein synthesis in HMC1 compared with CPA8 (p < 0.01; Fig. 5C).

The mechanism that underlies this inhibition of protein synthesis remains to be elucidated. It is interesting to note that in S. cerevisiae, polypeptide elongation factor-1α (55, 56) is a likely substrate for Nmt1p (N-terminal sequence = GKEKSHIN). The observed inhibition of protein synthesis by SC-61213 could reflect under-myristoylation of an orthologous C. neoformans protein which normally has a rapid turnover time or a perturbation in the interactions of Nmt with other ribosomal proteins (57).

Prospectus—Taken together, our results support the conclusion that the fungicidal effects of SC-61213 involve Nmt. A similar dose of SC-61213 has fungicidal activity against C. albicans Nmt (39), an observation that lends additional support for Nmt as a legitimate target for development of fungicidal drugs. Our isogenic C. neoformans strains, containing wild type C. neoformans or human Nmt, may prove useful for high throughput screens of large chemical or natural product libraries that seek to identify Nmt-based fungal-static or fungicidal compounds with relatively little activity against the human alycyltransferase.

Finally, the reagents developed during the course of this study may also be generally useful for genetic manipulations of C. neoformans. NMT1 is a good selectable marker; the phenotype produced by nmt487Δ is distinctive (pronounced temperature-sensitive myristic acid auxotrophy) and stable (reversion frequency <10−8). By using human as opposed to C. neoformans NMT1 as the selectable marker for transformation of CMD19, it should be possible to increase the copy number of integrated gene products. This approach of varying copy number by using orthologs may be applicable to other genes that are being employed as selectable markers in C. neoformans or other fungal pathogens.

Acknowledgments—We thank Dena Toffaletti, John Perfect, and Joe Heitman (all at Duke University) as well as Dwight Towler (Washington University) for their helpful suggestions and assistance.

REFERENCES
1. Mitchell, T. G., and Perfect, J. R. (1995) Clin. Microbiol. Rev. 8, 515–548
2. Powderly, W. G., Finkelstein, D. M., Feinberg, J., Frame, P., He, W., van der Horst, C., Koleta, S. L., Eyster, E., Carey, J., Waskin, H., Hooton, T. M., Hyslop, N., Spector, S. A., and Bezaette, S. A. (1995) N. Engl. J. Med. 332, 700–705
3. van der Horst, C. M., Saag, M. S., Cloud, G. A., Hamill, R. J., Graybill, J. R., Sobel, J. D., Johnson, P. C., Tsazon, C. U., Kortering, T., Moskovitz, B. L., Powderly, W. G., Dismukes, W. E., National Institute of Allergy and Infectious Diseases Mycoses Study Group, and AIDS Clinical Trials Group (1997) N. Engl. J. Med. 337, 15–21
4. Apsbaugh, J. A., Perfect, J. R., and Heitman, J. (1997) Genes Dev. 11, 3206–3217
5. Chang, Y. C., and Kwon-Chung, K. J. (1994) Mol. Cell. Biol. 14, 4912–4919
6. Chang, Y. C., Penoyer, L. A., and Kwon-Chung, K. J. (1996) Infect. Immun. 64, 1977–1983
7. Salas, S. D., Bennett, E. J., Kwon-Chung, K. J., Perfect, J. R., and Williamson, P. R. (1996) J. Exp. Med. 184, 377–378
8. Wickes, B. L., Mayorga, M. E., Edman, U., and Edman, J. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7327–7331
9. Perfect, J. R., Toffaletti, D. L., and Rude, T. H. (1993) Infect. Immun. 61, 4446–4451
10. Odom, A., Muir, S., Lim, E., Toffaletti, D. L., Perfect, J., and Heitman, J. (1997) EMBO J. 16, 2576–2589
11. Livi, L. L., Edman, U., Schneider, G. P., Greene, P. J., and Santi, D. V. (1994) Gene (Amst.) 150, 221–226
