Khafrefungin, a Novel Inhibitor of Sphingolipid Synthesis*

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In the course of screening for antifungal agents we have discovered a novel compound isolated from an endophytic fungus that inhibits fungal sphingolipid synthesis. Khafrefungin, which is composed of aldonic acid linked via an ester to a C22 modified alkyl chain, has fungicidal activity against Candida albicans, Cryptococcus neoformans, and Saccharomyces cerevisiae. Sphingolipid synthesis is inhibited in these organisms at the step in which phosphoinositol is transferred to ceramide, resulting in accumulation of ceramide and loss of all of the complex sphingolipids. In vitro, khafrefungin inhibits the inositol phosphoceramide synthase of C. albicans with an IC₅₀ of 0.6 nm. Khafrefungin does not inhibit the synthesis of mammalian sphingolipids, thus making this the first reported compound that is specific for the fungal pathway.

The incidence of human systemic fungal infections has increased dramatically in the last decade due to a rise in immunocompromised patients, including those receiving cancer chemotherapy and immunosuppressants, and in the human immunodeficiency virus infected population. Therapies are currently limited to a small number of compounds for the treatment of a rather diverse array of pathogenic fungi, which include Candida albicans and other Candida species, Cryptococcus neoformans, Aspergillus sp., and Histoplasma capsulatum. Each drug has limitations; toxicity is an issue with treatments based on amphotericin B, and resistance, which precluded the use of flucytosine as a stand-alone antifungal soon after its introduction, is now beginning to emerge as a problem with theazole and triazole class of inhibitors. Even with the most aggressive therapy that is available, the rate of mortality from aspergillosis is extremely high in some patient populations thus highlighting the need for the development of new treatments (1).

The sphingolipid biosynthetic pathway has been suggested as a good target for antifungal therapy (2). Although sphingolipids comprise a relatively small proportion of fungal phospholipids, they are essential. Saccharomyces mutants that do not make sphingolipids are not viable (3), and pathogenic fungi treated with inhibitors of sphingolipid synthesis are killed (4, 5). The initial steps in sphingolipid biosynthesis, from ketodihydrosphingosine synthesis through ceramide formation, are conserved in fungi and mammals except that fungi make phytosphingosine as their predominant sphingoid base with lesser amounts of dihydrosphingosine. Mammalian sphingolipids are composed of dihydrosphingosine and sphingosine. Fungi do not make sphingomyelin and instead transfer phosphoinositol to the C1 hydroxyl of ceramide to make inositol phosphoceramide (IPC). IPC is further modified by the addition of mannose to make mannosyl inositol phosphoceramide, and the addition of a second inositol diinositol phosphoceramide (6). Some fungi, including pathogenic species of Aspergillus, have been reported to make glucosylerceramide and lactosylerceramide (7), but the major sphingolipids of Candida, Cryptococcus, and Histoplasma are based on IPC (6, 8).

A number of inhibitors of the sphingolipid pathway that also have antifungal activity have been discovered, all from natural product sources. Three structurally distinct classes of inhibitors of serine palmitoyltransferase have been found: 1) the sphingofungin family, which includes sphingofungins A through F (4, 9) and myriocin/ISP1, which has immunosuppressant activity in addition to its antifungal activity (10); 2) the lipoxamycins, which are produced by actinomycetes (11); and 3) a newly described family of inhibitors called the viridiofungins (12). All of these compounds are very potent inhibitors (nanomolar to picomolar) of serine palmitoyltransferase, and though they kill a broad array of pathogenic fungi, they also inhibit the mammalian enzyme. Two types of inhibitors of the ceramide synthase have been described: the fumonisins, which are inhibitors of mammalian ceramide synthase (13) and have poor antifungal activity although they do inhibit the fungal enzyme; and ausstralifungin, which has very potent antifungal activity (5). The fumonisins are associated with severe toxicities in animals and possibly humans. Toxicity has been attributed not only to the loss of ceramide and complex sphingolipids, but also to the accumulation of the sphingoid base precursors of ceramide, which are components of signal transduction pathways (14, 15).

In this report we describe a novel inhibitor that shows specificity for fungal sphingolipids. Khafrefungin inhibits the IPC synthase of Saccharomyces cerevisiae and pathogenic fungi at picomolar to nanomolar concentrations but does not inhibit mammalian sphingolipid synthesis. Ceramide accumulates in response to khafrefungin treatment, and fungi are killed.

**EXPERIMENTAL PROCEDURES**

**Strains, Inhibitors, and Reagents—S. cerevisiae W303–1A (MATa, ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1) was provided by R. Rothstein (16). C. albicans MY1055, C. neoformans MY2062, and Aspergillus fumigatus MF5668 were obtained from the Merck Culture Collection (Rahway, NJ). For in vitro assays, C. neoformans strain Cap64 (ATCC52816) was used. Khafrefungin, viridiofungin A, and ausstralifungin were provided by G. Harris (Merck Research Laboratories). A. Rosegay, Y. S. Tang, and A. Jones (Merck Research Laboratories) synthesized [4,5-³H]dihydrosphingosine and [³H]ceramide. Acetylated...
sphingosine was exposed to tritium gas and palladium on carbon, deacetylated, and purified by HPLC to isolate (4,5,1H)dihydrosphingosine. N-stearoyl-D-sphingosine was incubated with NaB3H4, to synthesize N-stearoyl-β,(4,5,1H)dihydrosphingosine (β-creamide) that was purified by HPLC.

**Antifungal Assays**—Growth inhibition was determined by microtiter broth dilution assay in Difco yeast nitrogen base medium containing 2% glucose (YNBD) and 0.078% complete supplement mixture (CSM, purified by HPLC). The drug-treated cell suspension and spreading aliquots onto agar plates to monitor colony formation. The minimum fungal concentrations value was the lowest inhibitor concentration, which reduced the viable cell count by 99% of the inoculum level.

**Microtiter Assay for Sphingolipid Synthesis**—Cells were grown to 0.25 g/ml for the presence and absence of 0.4 Ci/ml [3H]inositol and dispensed into 96-well plates containing inhibitor. The plates were incubated at 30 °C (S. cerevisiae and C. neoformans) or 37 °C (C. albicans) for 120 min (S. cerevisiae) or 180 min (C. albicans and C. neoformans). The assay was terminated with the addition of trichloroacetic acid to 5%, and the plates were chilled at 4 °C for 20 min. Precipitated cells were harvested onto filters and total [3H]inositol incorporated into both sphingosylpyrophosphorylcholine (SPC) and sphingosylphosphorylcholine was quantitated in an LKB BetaPlate liquid scintillation counter (Wallac). The filters were removed from scintillant and washed with 0.1 N KOH in methanol/toluene (1:1) at room temperature for 60–120 min and washed sequentially in methanol, 5% trichloroacetic acid and twice more in methanol. The remaining radio-labeled material (alkali stable sphingolipids) was measured by counting the filters again, and the counts incorporated into PI were calculated by subtracting the sphingolipid counts from the total.

**Lipid Synthesis and TLC Analysis**—1-ml cultures of S. cerevisiae were grown to 0.25 ± 0.05 A550 in YNB medium at 30 °C and labeled with 2 μCi/ml [14C]acetate for 2 h in the presence and absence of 0.4 μg/ml khafrefungin, and extracted lipids were resolved by TLC in CHCl3, methanol, 2 N NH4OH at 1 ml/min on a Beckman Ultra Sphere 5 m ODS column (250 x 4.6 mm) fitted with a Phenomenex Sphere X 5 m C18 guard (30 x 4.6 mm). Absorbance was monitored at 254 nm, fractions were collected every 0.5 min, and radioactivity was quantitated by liquid scintillation counter.

**Microsomal Membranes**—Microsomal membranes were prepared from C. albicans, S. cerevisiae, and C. neoformans (Cap64) cultures grown to A550 of 1.0 as described (5). In vitro IPC synthase reactions (100 μl) contained 50 mM Tris-HCl, pH 7.0, 50 mM KCl, 0.05% sodium cholate, 5 μg of membrane protein, 25 μl PI, and traces of [3H]ceramide (stearyl-[1H]dihydrosphingosine, 0.18 mCi/ml) with or without inhibitor. Khafrefungin was added from an ethanol stock; the concentration of ethanol in the assay was less than 2% and did not affect IPC synthase activity. The substrates were combined with 0.1% β-octylglucoside and sonicated until clear (~10 s in a special ultrasonic cleaner, model G112SP1G, Laboratories Supplies Co.) and diluted 10-fold into the assay to initiate the reaction. The reaction was conducted at 22–25 °C for 1 h and quenched with 20 μl of a 5% (w/v) deoxycholate solution in water. [3H]IPC was separated from [3H]ceramide on small (0.5 ml bed volume) union exchange columns using Bio-Rad AG 4-X4 resin in 95% ethanol. The columns were equilibrated with formic acid and rinsed with water prior to use. The assay solution was applied to the column and [3H]ceramide was removed by washing the column with 20 ml of 95% ethanol. [3H]IPC was eluted with 1 M K-formate in 95% ethanol, mixed with 5 μl of Aquasol, and counted in a Beckman liquid scintillation counter. Controls were carried out with the medium devoid of enzymes, and all data were corrected for the radioactive blank.

**RESULTS**

**Antifungal Activity of Khafrefungin**—In the course of screening for new antifungal agents, an unidentified sterile fungus cultured from a Costa Rican plant sample was found to produce a potent inhibitor of sphingolipid synthesis. Isolation of the active component resulted in identification of the novel inhibitor khafrefungin. As shown in Fig. 1, khafrefungin is composed of aldonic acid ester linked to a C22 modified alky chain. Khafrefungin inhibited the growth of many species of yeast and filamentous fungi in agar diffusion assays and in liquid broth. The growth of C. albicans, C. neoformans, and S. cerevisiae in liquid culture was inhibited at khafrefungin concentrations of 1 μg/ml and higher, with minimum inhibitory concentrations of 2, 2, and 15.6 μg/ml for the three organisms, respectively (Fig. 2). Khafrefungin killed the fungi with minimum fucidial concentrations of 4, 4, and 15.6 μg/ml for C. albicans, C. neoformans, and S. cerevisiae, respectively. Examination of C. albicans and S. cerevisiae cells after drug treatment did not reveal any gross changes in morphology or unusual distribution of budded cells indicative of cell cycle arrest. A. fumigatus was not sensitive to growth inhibition by the inhibitor in liquid or in agar and the hyphal morphology was not affected even at high concentrations (250 μg/ml).

**Khafrefungin Inhibits Sphingolipid Synthesis**—The effect of khafrefungin on [3H]inositol incorporation into lipids of C. albicans was not sensitive to growth inhibition by the inhibitor in liquid or in agar and the hyphal morphology was not affected even at high concentrations (250 μg/ml).

![FIG. 1. The structure of khafrefungin.](image)

Eulsion was carried out at 40 °C with a methanol-acetonitrile-H2O gradient (75:25:25 to 95:2.5:2.5 over 60 min) at 2 ml/min. Absorbance was monitored at 242 nm, and 0.4 min fractions were collected across the gradient and counted by liquid scintillation counter.
bicans using a new microtiter-format assay, is shown in Fig. 3. Inositol is first incorporated into PI and then transferred to sphingolipids. These two fractions were distinguished by degrading the ester-linked PI with mild alkaline methanolysis; the remaining counts represent the amide-linked sphingolipids, which are resistant to saponification. Khafrefungin inhibited inositol incorporation into the sphingolipid fraction with an IC50 of 0.09 mg/ml (150 nM) but did not block inositol incorporation into PI. Compounds that inhibit sphingolipid synthesis at serine palmitoyltransferase (viridiofungin A) (12) or ceramide synthase (australifungin) (5) also specifically inhibited inositol incorporation into the sphingolipid fraction but were less potent than khafrefungin in this assay (Fig. 4).

Sphingolipid synthesis was tested in other organisms that were sensitive to growth inhibition by khafrefungin. Fig. 5 shows that khafrefungin inhibits sphingolipid synthesis in C. albicans (●), C. neoformans (□), and S. cerevisiae (▲) was measured as a function of khafrefungin concentration.

Hydrophosphingosine and lipid extracts were resolved by TLC. Fig. 6 is a fluorograph of lipids labeled with [3H]dihydrophosphingosine, which is incorporated most readily into the sphingolipid fraction. At a concentration that inhibited most of the label incorporation into IPC and mannosyl inositol phosphoceramide (200 ng/ml), khafrefungin did not affect the sphingoid base intermediates, which accumulate when ceramide synthesis is blocked, but did cause accumulation of a very nonpolar lipid that migrated slightly below the stearoyl-dihydrosphingosine standard. In the presence of khafrefungin, all of the sphingolipid precursors except [3H]inositol labeled this lipid, and simultaneous treatment with australifungin, the ceramide synthase inhibitor, prevented accumulation of this lipid (Fig. 6).

Thus, by virtue of its mobility, sensitivity to inhibitors, and substrate composition, the accumulating lipid was most likely to be hydroxyceramide, the predominant species of ceramide made by fungi.

We wanted to verify that the accumulating intermediate was ceramide and identify the sphingoid base and fatty acid components. [14C]acetate was used to label both components of ceramide, and the khafrefungin-dependent lipid that accumulated was isolated and hydrolyzed. Fractions were extracted, derivatized, and chromatographed by HPLC using methods designed to analyze long chain fatty acids (as their phenacyl derivatives) and sphingoid bases (as their biphenylcarbonyl derivatives). The accumulating intermediate was found to contain primarily a hydroxylated C24 fatty acid with small
amounts of hydroxy C26 and nonhydroxy C24 (Fig. 7A). In the sphingoid base analysis, the major component detected was C18 phytosphingosine with a second broad peak that comigrated with both C18 dihydrosphingosine and C20 phytosphingosine, which are only partially resolved by this system (20) (Fig. 7B).

By TLC analysis, which resolves dihydrosphingosine from phytosphingosine but does not separate species with different chain lengths, phytosphingosine was almost exclusively found (data not shown). Thus, khafrefungin causes the accumulation of hydroxyceramide with a composition that is consistent with a recent description of sphingolipids present in the hyphal form of *C. albicans* (20).

**Khafrefungin Inhibits IPC Synthase**—The accumulation of ceramide suggested that IPC synthesis might be the biosynthetic step that is blocked by khafrefungin. Inhibition of IPC synthase was confirmed in an *in vitro* enzyme assay. Previous assays for IPC synthase have relied on deacylation of [3H]PI substrate and chromatographic methods (21, 22) or differential organic extraction (2). An improved assay for IPC synthase was developed that uses a simple ion exchange procedure to separate [3H]ceramide from [3H]IPC. The assay has a precision of ±3% standard error with background radioactivity less than 0.4%, and it is suitable for large scale screening. IPC synthase, being membrane bound and operating on membrane components as substrates, is not amenable to standard kinetic analysis, primarily because the concentration of substrates in crude membranes is difficult to control. Although IPC synthase can be assayed by adding trace [3H]ceramide to a membrane containing endogenous PI, the rates of incorporation are slow and exogenous PI has little influence. Cholate, almost alone among a number of detergents tested, markedly enhanced the catalytic activity and extent of incorporation of [3H]ceramide at concentrations below or approaching its critical micelle concentration and was adopted as a component in subsequent assays. Nonetheless, the reaction remained a complex function of PI, ceramide, and detergent concentrations. In the standard assay, khafrefungin was found to be a very potent inhibitor of the *C. albicans* enzyme with an IC50 of 0.6 nM as shown in Fig. 8. The IPC synthase enzymes from *S. cerevisiae* and *C. neoformans* were 10- and 50-fold less sensitive with IC50 values of 7 and 31 nM, respectively. These values appeared relatively insensitive to changes in substrate or detergent concentration, but increased linearly with increasing enzyme-membrane concentration.

**Khafrefungin Does Not Inhibit Mammalian Sphingolipid Synthesis**—Known inhibitors of serine palmitoyltransferase and ceramide synthase have approximately the same potency against fungal and mammalian enzymes. These compounds also inhibit mammalian sphingolipid synthesis in intact cells, as described below. We sought to determine whether khafrefungin inhibits mammalian sphingolipid synthesis given that fungal IPC synthase differs significantly from its mammalian counterpart in substrate specificity. Sphingomyelin synthase is the analogous enzyme in the mammalian pathway; it transfers phosphocholine to ceramide to make sphingomyelin. HepG2 cells were labeled with [3H]-serine and lipids extracted, deacylated, and separated by TLC (Fig. 9). Viridiofungin (5 μM) prevented serine incorporation into sphingomyelin and ceramide, but did not affect serine incorporation into phosphatidylserine.
or phosphatidylethanolamine, which are converted to glycerophosphoserine and glycerophosphoethanolamine by alkaline transacylation. With australifungin treatment (10 μM), ceramide and sphingomyelin synthesis were partially inhibited, and 3H-serine incorporation was enhanced into several lipids, including dihydrosphingosine, glycerophosphoethanolamine, and a lipid comigrating with the nonpolar species of sphingomyelin which may be sphinganine 1-phosphate. These results with australifungin are the expected consequence of ceramide synthase inhibition, based on observations with fumonisin B1, which has been shown to promote 3H-serine incorporation into sphingoid bases and their degradation products (23). In contrast to these compounds that inhibit enzymes in the initial stages of sphingolipid synthesis, khafrefungin did not inhibit serine incorporation into sphingomyelin or any mammalian lipid at a concentration that was more than 300-fold above that required to inhibit IPC synthesis. Thus, khafrefungin is the first inhibitor described that is specific for fungal sphingolipid synthesis.

**DISCUSSION**

*S. cerevisiae* has been found to be surprisingly flexible in terms of its requirements for phospholipids. The phospholipid composition varies dramatically in different growth conditions and mutant studies have been particularly revealing about which lipids are essential (24, 25). For instance, studies on mutants that lack phosphatidylserine synthase have resulted in the discovery that phosphatidylserine is dispensable for growth (although other processes are impaired), and analysis of mutants defective in fatty acid elongation have resulted in the discovery that phosphatidylserine is dispensable for growth (although other processes are impaired). Sphingolipid synthesis impedes processing of glycosylphosphatidylinositol anchors on mannoproteins that constitute a major component of the yeast cell wall and sphingolipid synthesis.

Sphingolipids, although present in relatively low abundance, are also essential, and inhibition of sphingolipid synthesis is lethal. Like PI, sphingolipids play multiple roles in yeast, any one of which could serve their essential function. Sphingolipids and their intermediates appear to be lipid-signaling molecules in mammalian cells where they regulate key enzymes involved in growth and differentiation (15, 27–29). They may play a similar role in *Saccharomyces*; some of the components of a ceramide signal transduction pathway have been identified and ceramide has been shown to activate a protein phosphatase and mediate G1 arrest (30). Sphingolipid bases also have a regulatory role in yeast where they have been shown to inhibit several key enzymes in phospholipid biosynthesis when induced to accumulate by fumonisin treatment (31). Sphingolipids are involved in two ways with the synthesis of glycosylphosphatidylinositol anchors on *Saccharomyces*; a substantial proportion of mature glycosylphosphatidylinositol-anchored proteins are composed of IPC instead of PI in what appears to be a remodeling step (32), and inhibition of sphingolipid synthesis impedes processing of glycosylphosphatidylinositol-anchored proteins through the secretory pathway (33, 34). Finally, sphingolipids appear to be the major, and perhaps sole repository for very long chain fatty acids (C24 and C26 species) in fungi. Mutants defective in fatty acid elongation are impaired in sphingolipid synthesis and have pleiotrophic defects in the activities of several different enzymes, in transcriptional regulation, and in the sterol and endocytic pathways (17). Very long chain fatty acids have recently been shown to be important in nuclear pore formation (35), and they may have a role in membrane budding and fusion (36); functions which if im-

**FIG. 8. Khafrefungin inhibits IPC synthesis in vitro.** IPC synthase activity was measured using 3H-ceramide substrate and anion exchange chromatography in membrane extracts from C. albicans (○), C. neoformans (●), and S. cerevisiae (▲) with varying concentrations of khafrefungin. The dots are theoretical for a fit of the data to the equation y = a(1 + (x/c)^h), where a is the maximum velocity in the absence of inhibitor; b is the Hill coefficient, which is fixed with a value of 1; and c represents the calculated IC_{50}.

**FIG. 9. Mammalian sphingolipid synthesis is not inhibited by khafrefungin.** HepG2 cells were labeled with [3H]serine in the presence of 10 μM australifungin (lane 1); methanol (lane 2); 5 μM viridofungin A (lane 3); 50 μM khafrefungin (lane 4); or 10 μM khafrefungin (lane 5). Lipids were extracted, deacylated, separated by TLC, and visualized on x-ray film following treatment with EN^3HANCE. Lipid standards: CER, stearoyl dihydrosphingosine; DHS, dihydrosphingosine; SM, sphingomyelin; GPE, glycerophosphoethanolamine; GPS, glycerophosphoserine.
in vitro tested, C. albicans whether khafrefungin is a competitive inhibitor due to the phoinositol. Unfortunately, we have been unable to test confers fungal specificity by virtue of its resemblance to phos-enzymes with equal potency, khafrefungin did not inhibit role of sphingolipids in studies with inhibitors and mutants may help to determine the also reported to contain glycosylated sphingolipids (7). Future sphingolipids may not be essential in this organism, which is uptake of the drug. Alternatively, the inositol-containing growth inhibition may be due to a resistant enzyme or poor does synthesize alkali-stable inositol lipids, and A. the growth of this pathogen (5, 38). We have found that pergillus aureobasidin. Sphingolipids are thought to be essential in pathogen, a fungid does not have antifungal activity against an important response to exogenously added phytosphingosine (2). Khafrefun-gin does not have antifungal activity against an important pathogen, A. fumigatus, a limitation that is also shared by aureobasidin. Sphingolipids are thought to be essential in As-pergillus, as inhibitors at earlier steps in the pathway inhibit the growth of this pathogen (5, 38). We have found that A. fumigatus does synthesize alkali-stable inositol lipids, and their synthesis is inhibited by khafrefungin but at much higher concentrations than those required for the other fungi. Lack of growth inhibition may be due to a resistant enzyme or poor uptake of the drug. Alternatively, the inositol-containing sphingolipids may not be essential in this organism, which is also reported to contain glycosylated sphingolipids (7). Future studies with inhibitors and mutants may help to determine the role of sphingolipids in Aspergillus.

Unlike reported inhibitors of serine palmitoyltransferase and ceramide synthesis which affect fungal and mammalian enzymes with equal potency, khafrefungin did not inhibit mammalian sphingolipid synthesis. Since mammalian sphin-gomyelin synthase and fungal IPC synthase both use ceramide as a substrate, it is tempting to speculate that the highly hydroxylated acidic polar headgroup on khafrefungin (Fig. 1) confers fungal specificity by virtue of its resemblance to phos-phoinositol. Unfortunately, we have been unable to test whether khafrefungin is a competitive inhibitor due to the complexities of the in vitro enzyme reaction that employs a crude membrane preparation, detergent, two lipid substrates, and a hydrophobic inhibitor that partitions into the lipid fraction. More rigorous kinetic analysis awaits purification of the enzyme, which may be facilitated by the recent identification of the IPC synthase gene in Saccharomyces (2). Khafrefungin should provide a useful tool to manipulate ceramide levels and probe the function of ceramide in fungal cell growth, differentiation, and stress response pathways.

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