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Disruption of the glucosylceramide biosynthetic pathway in *Aspergillus nidulans* and *Aspergillus fumigatus* by inhibitors of UDP-Glc:ceramide glucosyltransferase strongly affects spore germination, cell cycle, and hyphal growth

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Abstract The opportunistic mycopathogen *Aspergillus fumigatus* expresses both glucosylceramide and galactosylceramide (GlcCer and GalCer), but their functional significance in *Aspergillus* species is unknown. We here identified and characterized a GlcCer from *Aspergillus nidulans*, a non-pathogenic model fungus. Involvement of GlcCer in fungal development was tested on both species using a family of compounds known to inhibit GlcCer synthase in mammals. Two analogs, D-*threo*-1-phenyl-2-palmitoyl-3-pyrrolidinopropanol (P4) and D-*threo*-3′,4′-ethylenedioxy-P4, strongly inhibited germination and hyphal growth. Neutral lipids from *A. fumigatus* cultured in the presence of these inhibitors displayed a significantly reduced GlcCer/GalCer ratio. These results suggest that synthesis of GlcCer is essential for normal development of *A. fumigatus* and *A. nidulans*. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Sphingolipid; Fungus; Glycosyltransferase; Glycolipid; PDMP; Synthase

1. Introduction

Investigations of glycosphingolipid (GSL) biosynthesis and function in *Saccharomyces cerevisiae* and other fungi have established that they are essential components of the fungal cell membrane [1–3]. These studies have focused mainly on glycosylinositol phosphorylceramides (GIPCs), a family of compounds widely distributed among fungi but not found in animal cells or tissues [4,5]. Unlike *S. cerevisiae*, however, most fungi synthesize not only GIPCs, but also neutral monohexosylceramides (also referred to as cerebrosides or CMHs). It appears that the majority of fungi express glucosylceramide (GlcCer) only, but some Euascomycetes, such as *Aspergillus fumigatus* [6,7], *Aspergillus niger* [8], and *Sporothrix schenckii* [9], also synthesize galactosylceramide (GalCer). The ceramide moieties in fungal cerebrosides have a number of structural features that distinguish them from those found in mammalian GSLs, including (E)-Δ^8 unsaturation and 9-methyl group branching of the sphingoid base, and variable levels of (E)-Δ^3 unsaturation of the fatty acid. In addition, they are different from those found in fungal GIPCs, suggesting that they are assembled from structurally distinct pools of sphingoid bases [1,7].

Several putative GlcCer synthase (GCS; UDP-Glc:ceramide β-glucosyltransferase) gene homologs have appeared in fungal genome databases. A number of these were cloned, expressed, and their enzymatic function verified [10]. However, despite a number of studies demonstrating intriguing physiological activities of endogenously added fungal cerebrosides [11–14], the true in vivo functions of these compounds remain unclear. Structural analogs of D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-*threo*-PDMP) [15] have been widely applied for inhibition of GCS in mammalian cells [16–18], but no applications to fungi have been reported. Recently, PDMP analogs have been generated exhibiting improved specificity and potency, eliminating undesired effects encountered with the parent compound, such as ceramide accumulation and resultant triggering of apoptosis [16,17]. The studies described in this paper demonstrate that some PDMP analogs strongly affect cerebroside biosynthesis in *A. fumigatus* and *Aspergillus nidulans*, resulting in profound physiological effects.

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Abbreviations: ESI, electrospray ionization; HPTLC, high performance thin layer chromatography; GSL, glycosphingolipid; CMH, ceramide monohexoside (≡cerebroside); GlcCer, β-glucopyranosylceramide (≡glucocerebroside); GalCer, β-galactopyranosylceramide (≡galactocerebroside); GIPC, glycosylinositol phosphorylceramide; GCS, glucosylceramide synthase; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol

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2. Materials and methods

2.1. GCS inhibitors

Inhibition studies were performed with D/L-*threo*-PDMP [15], and a series of homologs and analogs of the active isomer D-*threo*-PDMP: D-*threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) [19], D-*threo*-1-phenyl-2-palmitoyl-3-pyrrolidinopropanol (PPPP or P4) [16,17], and D-*threo*-3′,4′-ethylenedioxy-P4 (EDO-P4) [17].

2.2. Fungal isolates and culture conditions

A. fumigatus ATCC strain 9197 and *A. nidulans* FGSC strain A28 were used for most experiments. *A. fumigatus* strain 237, originally cultured from open lung biopsy from a patient with invasive pulmonary aspergillosis at Hope Hospital (Manchester, UK), was the gift of Dr. David W. Holden (Hammersmith Hospital, London, UK). Approximately 1×10^9 spores were inoculated to 200 ml complete medium (1% glucose, 0.2% peptone, 0.1% yeast extract, 0.1% casamino acids, nitrate salts, trace elements, and 0.01% vitamins, pH 6.5; trace elements, vitamins, nitrate salts, and amino acid supplements are described in the appendix to Kafer [20]), incubated at 30 or 37°C with shaking (200 rpm) for 24 h, filtered, and processed as described. Yields were 3–10 g wet weight per 200 ml medium. For experiments in which mycelia were grown in the presence of GCS inhibitors, incubations were performed at 37°C, and inhibitors were added from 70% ethanol stock solutions 6 h after inoculation.

2.3. Solvents for extraction, anion exchange chromatography, and HPTLC

Solvent A, chloroform/methanol (1:1, v/v); solvent B, isopropanol/hexane/water (55:25:20 v/v, upper phase discarded); solvent C, chloroform/methanol/water (30:60:8 v/v); solvent D, chloroform/methanol/2 N ammonium hydroxide (50:10:1 v/v); solvent E, isopropanol/hexane/water (55:40:5 v/v).

2.4. Extraction, purification, and characterization of GSLs

Extraction and purification of GSLs were carried out as described previously [7]. Briefly, GSLs were extracted by homogenizing mycelia (3–10 g wet weight) in an Omni-mixer (Sorvall, Wilmington, DE, USA), once with 100 ml of solvent A, twice with 100 ml of solvent B, and once more with 100 ml of solvent A. Between homogenization steps, mycelia were collected by vacuum filtration through a sintered glass funnel. The four filtrates were pooled, dried on a rotary evaporator, dialyzed against water, lyophilized, resuspended in solvent C, and applied to a column of DEAE-Sephadex A-25 (Ac^- form). Neutral lipids containing CMHs were eluted with five volumes of solvent C, dried down, and examined by high performance thin layer chromatography (HPTLC) as described below; where necessary, individual components were further purified by preparative-scale HPTLC as previously described [21]. The purity and identity of each fraction were assessed by analytical HPTLC, ^{1}H-NMR spectroscopy, and positive ion mode electrospray ionization mass spectrometry ($^+$ESI-MS) as described previously [7–9,21].

2.5. Analytical HPTLC

All HPTLC was performed on silica gel 60 plates (E. Merck, Darmstadt, Germany) using solvent D as mobile phase [21]. Samples were dissolved in solvent E and applied by streaking from 10 µl Micro-caps (Drummond, Broomall, PA, USA). For analytical HPTLC, detection was made by Bial's orcinol reagent (orcinol 0.55% w/v and H_2SO_4 5.5% v/v in ethanol/water 9:1 v/v; the plate is sprayed and heated briefly to ∼200–250°C).

2.6. Coverslip assays, staining and microscopy

The protocol of Harris et al. [22] was used for growth and staining of *A. fumigatus* and *A. nidulans* as follows. Ten ml complete liquid medium was inoculated with $1–5 \times 10^4$ conidia/ml, poured into a Petri dish containing a glass coverslip, and incubated at 37°C (in some cases 30°C) for the time indicated in each experiment. Where used, GCS inhibitors were added at $t = 5$ h after the start of incubation unless otherwise noted. Coverslips with adhering germlings were fixed in 3.7% formaldehyde, 50 mM phosphate buffer (pH 7.0) and 0.2% Triton X-100 for 30–60 min. Coverslips were then washed with water, incubated 5 min with 10 µg/ml Calcofluor (Bayer, Leverkusen, Germany) and 100 ng/ml Hoechst 33258 (Sigma), washed again and mounted on a microscope slide for viewing. Germlings were observed using a Zeiss Axioplan microscope and photographed using an Optronics Digital Imaging System (Goleta, CA, USA).

2.7. Minimal inhibitory concentration experiments

Approximately 10^3 conidia were inoculated to 1 ml complete medium, and inhibitors added from a concentrated stock in 70% ethanol. Negative control samples contained an equivalent amount of 70% ethanol. After incubation at 37°C without agitation for 3 h, conidia were washed twice in fresh complete medium, resuspended in complete medium and plated. After 36 h incubation at 37°C, colony forming units were counted. The experiment was repeated twice with similar results.

3. Results

3.1. Comparison of GlcCer and GalCer expression in *A. fumigatus* and *A. nidulans*

It is known that *A. fumigatus* expresses both GlcCer and GalCer, and these components have been thoroughly characterized [6,7]. In the present work, we observed quantitative variability of these components depending on strain and culture conditions, especially temperature. Two strains of *A. fumigatus* (9197 and 237) were cultured for 24 h at 30°C or 37°C, under otherwise identical conditions, and their total neutral lipids compared by HPTLC analysis (Fig. 1, lanes 1–4). Bands corresponding to GlcCer and GalCer were resolved and identified by co-migration with standard compounds previously isolated and characterized [7] (lanes S1, S2, respectively). In both strains of *A. fumigatus*, the relative amounts of GlcCer were elevated at 37°C (lanes 2 and 4). Since the GlcCer/GalCer ratio was observed to be highest with *A. fumigatus* strain 9197 at 37°C, subsequent experiments with inhibitors were performed with this strain, and measurements of GlcCer/GalCer ratios were carried out at 37°C.

In *A. nidulans*, orcinol-positive bands corresponding in R_f value to GlcCer were observed at both temperatures (Fig. 1, lanes 5 and 6). Since GSLs of *A. nidulans* have never been characterized, the putative GlcCer components from both culture temperatures were isolated by preparative HPTLC and subjected to structural analysis by ^{1}H-NMR and $^+$ESI-MS.

3.2. Characterization of *A. nidulans* cerebroside fractions

Both ^{1}H- and ^{13}C-NMR spectra for underivatized fungal cerebrosides have been previously acquired and all resonances assigned by homonuclear and heteronuclear 2-D correlation

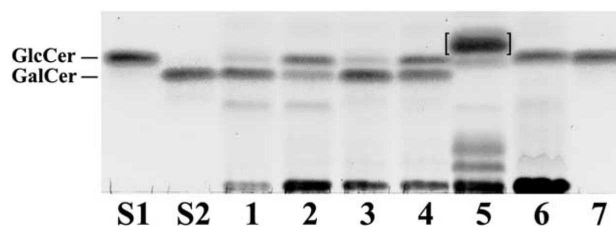

Fig. 1. Comparison of GlcCer and GalCer expression in *A. fumigatus* and *A. nidulans* showing temperature dependence. Lower section of orcinol-stained analytical HPTLC plate compares crude neutral lipids extracted from *A. fumigatus* strains 9197 (lanes 1,2) and 237 (lanes 3,4), and *A. nidulans* strain A28 (lanes 5,6). Mycelia were cultured at 30°C (lanes 1,3,5) or 37°C (lanes 2,4,6). Lanes S1 and S2, standards of GlcCer and GalCer from *A. fumigatus* strain 9197 previously characterized by NMR and $^+$ESI-MS [8]. Bracketed band appearing in *A. nidulans* strain A28 only at 30°C (lane 3) is sterol glucoside. Lane 7, putative GlcCer purified from *A. nidulans*, 37°C culture.

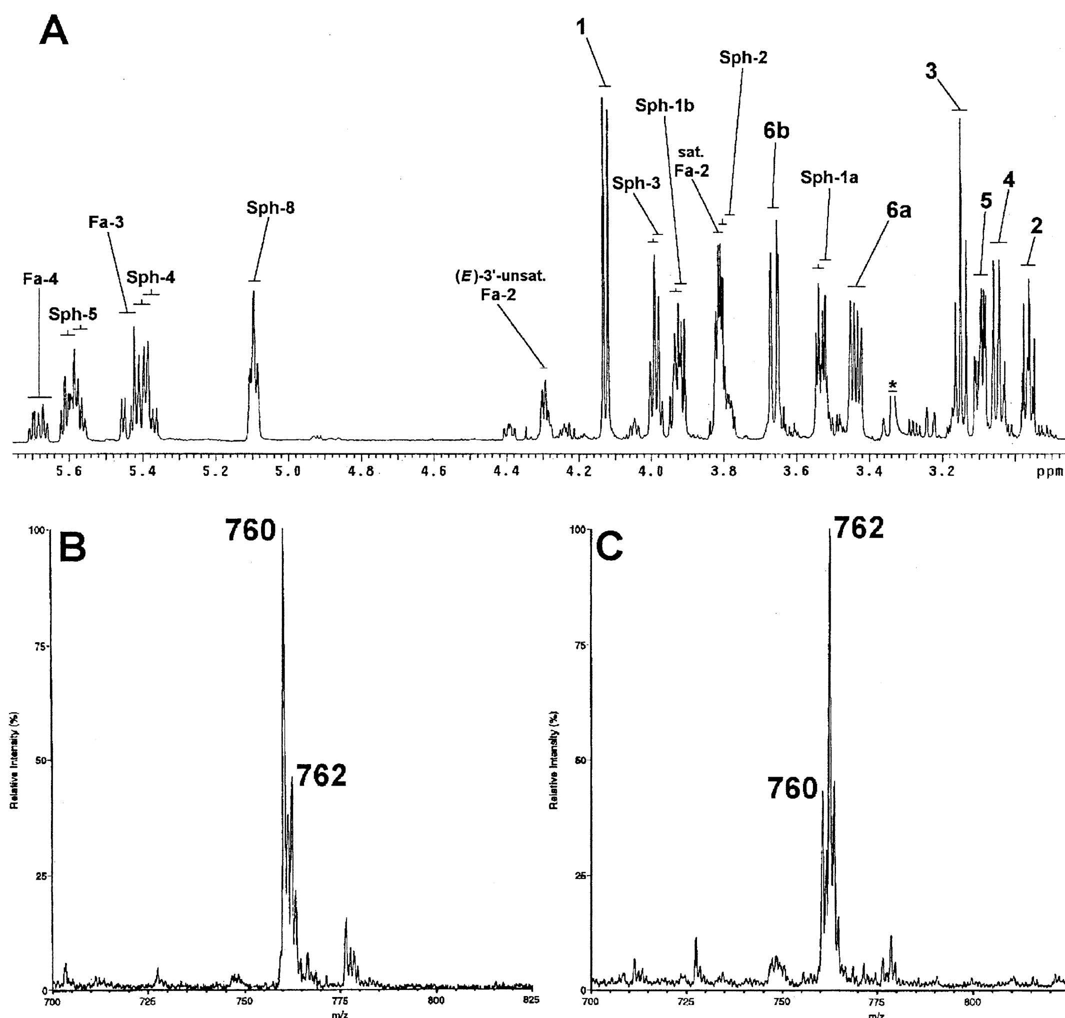

Fig. 2. Structural characterization of *A. nidulans* cerebrosides. A: Downfield section of 1-D ^{1}H-NMR spectrum (DMSO-d_6/2% D_2O, 35°C) of CMH isolated from *A. nidulans* cultured at 37°C. Resonances from non-exchangeable protons of sphingosine (Sph), fatty acyl (Fa), and hexose (prefix omitted) are designated by arabic numerals. Two locations each are shown for Sph-1a,1b to Sph-5 and Fa-2, corresponding to components having saturated or (*E*)-3′-unsaturated 2-hydroxy fatty acid. B,C: $^+$ESI-MS molecular ion profiles of CMH (as Li^+ adducts) isolated from *A. nidulans* mycelia cultured at 30°C and 37°C, respectively.

methods [7,21]. For the present work it was therefore sufficient to obtain 1-D ^{1}H-NMR spectra of putative *A. nidulans* GlcCer fractions under identical conditions in order to confirm their structures, except for the absolute lengths of the ceramide alkyl/acyl chains. The representative spectrum in Fig. 2A, obtained from the 37°C *A. nidulans* cerebroside, exhibits all seven ^{1}H resonances characteristic of the β-glucopyranosyl spin system, as well as resonances for key ceramide features observed previously for fungal cerebrosides [7]. These ceramide resonances indicate the presence of two predominant ceramide types, consisting of [9,21] a (4*E*,8*E*)-9-methyl-4,8-sphingadienine *N*-acylated with either 2′-hydroxyalkanoate or 2′-hydroxy-(*E*)-3′-alkenoate. No resonances from GalCer were detected in any *A. nidulans* cerebroside spectra.

In $^+$ESI-MS (Fig. 2B,C), abundant monolithiated molecular ion adducts were observed for both GlcCer fractions at *m/z* 760 and *m/z* 762. These adduct masses are consistent with GlcCer containing (d19:2) (4*E*,8*E*)-9-methyl-4,8-sphingadienine attached to *N*-2′-hydroxy-(*E*)-3′-octadecenoate or *N*-2′-hydroxyoctadecanoate, respectively. Interestingly, the considerable predominance of the low *m/z* component at 30°C (Fig. 2B) is reversed at 37°C (Fig. 2C). In product ion spectra obtained from the lithiated molecular ions (*m/z* 760 or *m/z* 762) by tandem $^+$ESI-MS/collision-induced dissociation-MS (not shown), the masses and relative abundances of all fragments were in each case virtually identical with those obtained previously for GlcCer with the proposed ceramides differing only in the absence or presence of (*E*)-3′-unsaturation [8,21].

3.3. Effects of GCS inhibitors on growth and morphology of *A. nidulans* and *A. fumigatus*

The effects of GCS inhibitors were initially characterized by observation of germination and subsequent hyphal growth of *A. nidulans* and *A. fumigatus* spores in the presence or absence of several PDMP analogs. When the round, asexual spores (conidia) are introduced into nutrient medium, they break dormancy and begin to grow isotropically, adding new material uniformly to the cell periphery. After a defined period of time (5 h under our conditions), polar growth is established with new material being added to only one region of the cell surface. The result is the emergence of a germ tube and tip elongation typical of filamentous fungi. PDMP analogs were

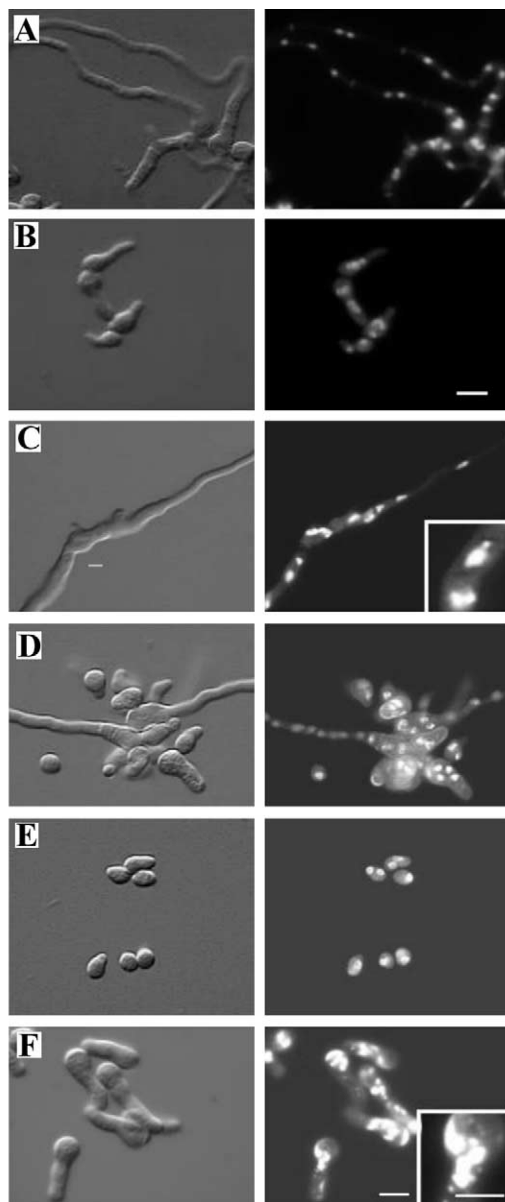

Fig. 3. Effect of GCS inhibitors on early growth of *A. nidulans* and *A. fumigatus*. Conidia were inoculated to rich medium, inhibitor was added after 5 h incubation at 37°C, and incubation continued for 5 h more prior to fixing and staining with Calcofluor white and Hoechst 33258 to visualize cell walls and nuclei, respectively. *A. nidulans*: A: no inhibitor; B: EDO-P4, 20 μM. *A. fumigatus*: C: no inhibitor; D: P4, 20 μM; E: EDO-P4, 20 μM; F: EDO-P4, 4μM. Insets, enlargement of nuclei to illustrate nuclear morphology. Left column, DIC image; right column, fluorescence image. Scale bars = 10 μm.

added to conidia after 5 h of incubation, at the point where they typically have become fully swollen and begin to grow asymmetrically as germ tube growth is initiated [23]. Incubation was continued for 5 more hours prior to fixing and staining, which is sufficient for extensive hyphal elongation, as shown for the controls in Fig. 3A,C. Addition of PDMP or PPMP at 20 μM had no detectable effect on either species (not shown). On the other hand, addition of EDO-P4 at 20 μM to *A. nidulans* conidia halted growth just after germ tube emergence (Fig. 3B).

We considered that the ability of *A. fumigatus* to synthesize an alternative cerebroside (GalCer) might ameliorate the effect of GCS inhibitors. However, compared with the control, addition of P4 at 20 μM still severely limited growth of hyphae, with most cells arresting growth just after polarity establishment (Fig. 3D). Addition of EDO-P4 at 20 μM halted growth before germ tube emergence (Fig. 3E). Although these results are from experiments carried out at 37°C, similar observations were made for both species at 30°C.

Addition of P4 or EDO-P4 to either species at a lower concentration (4 μM) resulted in macroscopically normal growth, but microscopic examination revealed nuclear abnormalities. As shown for *A. fumigatus* in Fig. 3F, many nuclei appeared globular and of variable sizes relative to controls. Even in cells where nuclei looked normal, there appeared to be a higher number of mitotic nuclei. The mitotic index rose from 4% in untreated *A. fumigatus* controls to 28–34% in cultures treated with 4 μM P4 or EDO-P4. Thus, in addition to observed morphological effects, PDMP analogs might affect DNA replication and segregation.

In the preceding experiments, inhibitor was added after spores had broken dormancy and completed most of their isotropic growth phase. To determine if PDMP analogs are also effective at other points in the early growth of *A. fumigatus*, 40 μM EDO-P4 was added to cultures at defined time points during incubation, beginning with $t = 0$. All cultures were fixed and stained at the same end point, $t = 10$ h. In parallel, untreated control samples were fixed and stained at each time point, reflecting the normal morphological states observable during early fungal growth. As shown in Fig. 4, cells which were incubated after addition of EDO-P4 for sufficient time to exhibit extensive hyphal growth appeared identical to untreated controls which were fixed at the time of inhibitor addition. Thus, the arrest of early fungal growth by EDO-P4 is not stage-specific.

3.4. Minimal inhibitory concentration experiments

To determine if *A. fumigatus* and *A. nidulans* were able to recover from drug-induced growth arrest, a colony forming assay was performed. Spores of *A. fumigatus* or *A. nidulans* were treated with inhibitor, washed, plated to solid medium and incubated for 2 days. The concentration of P4 or EDO-P4 was varied to provide a dose-dependent measure of response. As shown in Fig. 5, a large percentage of spores were viable at an inhibitor concentration of 0.4 μM, but virtually none were viable after treatment with inhibitor at 40 μM. From these results it is estimated that the IC_{50} in this assay for EDO-P4 is 1–2 μM, while that for P4 is 0.5–1 μM.

3.5. Effects of GCS inhibitors on GlcCer/GalCer expression in *A. fumigatus*

In order to verify that GCS inhibitors developed for the mammalian enzyme could also affect GlcCer synthesis in fungi, lipids were extracted from fungal cells grown in the presence or absence of sublethal doses of inhibitors, and analyzed by HPTLC. Growth in the presence of inhibitors was accomplished by inoculation with a higher spore load than employed in the coverslip assay (by a factor $> 10^3$ per ml), and delaying the addition of drug until germ tube extension was well under way. The experiment was performed with *A. fumigatus*, as its ability to synthesize GalCer as well allowed the effects of inhibition to be observed more easily via alteration of the *ratio* of the two cerebroside components.

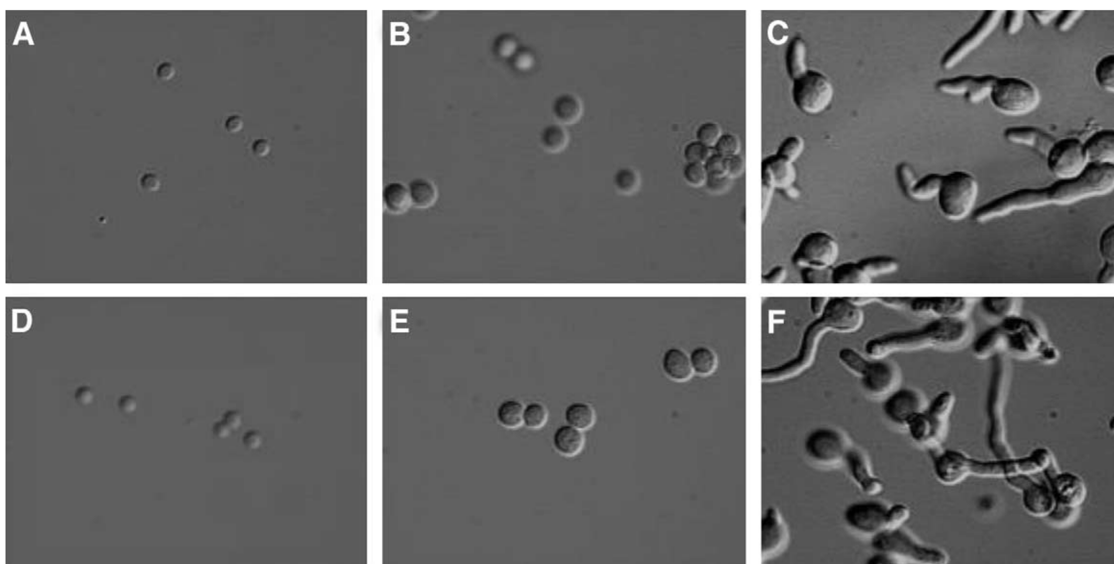

Fig. 4. Effect of GCS inhibitor EDO-P4 on *A. fumigatus* growth. *A. fumigatus* conidia were inoculated to rich medium, 40 μM EDO-P4 was added at defined time points, cells were incubated until $t = 10$ h, fixed and stained with Calcofluor white and Hoechst 33258 to visualize cell walls and nuclei, respectively. Controls were fixed at time of drug addition. A–C: Untreated control; D–F: EDO-P4 treated; A and C: 0 h; B and E: 4 h; C and F: 7 h.

Compared with the control grown without inhibitor, mycelia grown in the presence of 20 μM P4 and EDO-P4 clearly exhibited an increase in the amounts of GalCer compared with GlcCer (Fig. 6, lanes 4 and 6). P4 at 4 μM also appeared to have some effect (lane 3). Although these HPTLC profiles are semi-quantitative, at least in the case of 20 μM EDO-P4 the increase in staining of GalCer is clearly accompanied by a concomitant decrease in staining of GlcCer (lane 6). PDMP and PPMP appeared to have little or no effect on the amount of GalCer expressed (lanes 1 and 2, respectively).

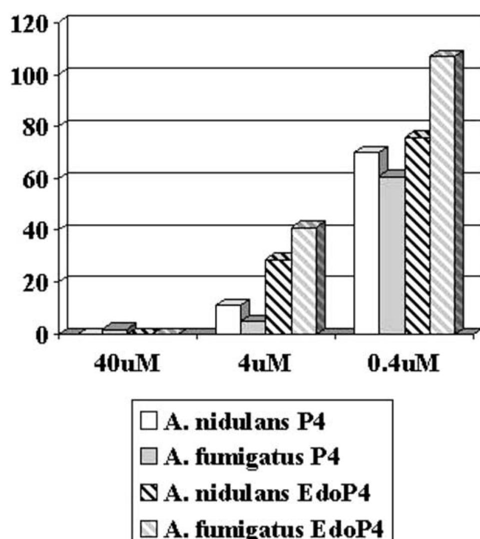

Fig. 5. Colony forming assay on *A. fumigatus* conidia treated with P4 and EDO-P4. Conidia were treated with inhibitors at 40, 4, and 0.4 μM, washed thoroughly, and replated on solid agar medium. Number of colony forming units after 48 h incubation relative to untreated controls is shown.

4. Discussion

With only a few exceptions – notably the common laboratory strains of *S. cerevisiae* and *Schizosaccharomyces pombe* – the biosynthetic machinery for GlcCer expression appears to be universally distributed among fungi [1,8,10,21,24] (and references cited therein). Genes encoding proteins exhibiting significant homology to mammalian GCSs can be found in several fungal genomic databases, including those of *Candida albicans* and *Neurospora crassa*. Although the identities in peptide sequence alignments are low (16–21% for *C. albicans*, *Magnaporthe grisea*, and *Pichia pastoris* compared to human) [10], the similarities increase considerably if conservative substitutions are included. In any case, the biosynthetic functions of the *C. albicans*, *M. grisea*, and *P. pastoris* homologs were recently demonstrated by cloning and expression in *S. cerevi-*

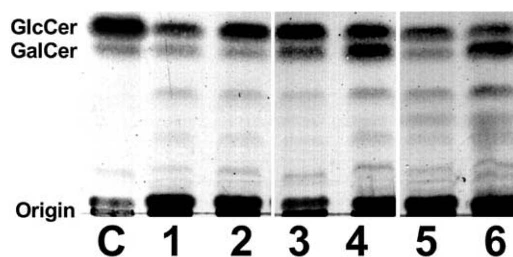

Fig. 6. Effects of GCS inhibitors on relative levels of GlcCer and GalCer expression in *A. fumigatus*. Shown is lower section of orcinol-stained analytical HPTLC plate comparing crude neutral lipids extracted from *A. fumigatus* strain 9197 cultured at 37°C grown in the absence of inhibitor (lane C, control), and in the presence of PDMP at 20 μM (lane 1), PPMP at 20 μM (lane 2), P4 at 4 and 20 μM (lanes 3 and 4, respectively), and EDO-P4 at 4 and 20 μM (lanes 5 and 6, respectively). Amounts streaked were normalized by adjusting the dilution with respect to wet weight yields of mycelia extracted (125 μl solvent per g mycelium; 10 μl per lane). Note that two pairs of lanes, 3 and 4, 5 and 6, have been digitally 'flipped' with respect to the original plate.

siae or in a GCS null mutant generated from *P. pastoris* [10]; successful expression was verified by isolation and characterization of authentic GlcCer products from the previously null yeast strains.

While isolation and analysis of GlcCer from fungi is straightforward, direct assays of GCS activities [25,26] have so far failed in our hands to work with fungal membrane extracts. As far as we are aware, no successful adaptation of a GCS assay to a fungal homolog has been reported. Similar to Leipelt et al. [10], we therefore relied on direct analysis of the cerebroside products from *A. fumigatus* grown in the presence or absence of sublethal amounts of inhibitors as evidence for their effects on GlcCer biosynthesis. These experiments clearly showed buildup of GalCer in the presence of P4 and EDO-P4, consistent with inhibition of a GCS that is competing for the same pool of ceramide acceptor substrate. The most striking quantitative differences correlated with inhibitors having the greatest potency and specificity toward the mammalian enzyme [16,17], and these in turn correlated with the most dramatic effects observed in the biological assays. Too little is known at this time to assume that no other enzymes are directly affected by these compounds in fungi, but the observed trends are consistent with the designed and well-tested properties of P4 and EDO-P4, having GCS as their sole target [16,17].

Interestingly, the susceptibility of *A. fumigatus* appears to be similar to that of *A. nidulans*, which does not produce GalCer. The effects of P4 and EDO-P4 on *A. fumigatus* and *A. nidulans* suggest that such inhibitors of GCS will be useful tools for analyzing the physiological function(s) of GlcCer and GlcCer synthase in fungi. In addition, as has already proved the case with inositol phosphorylceramide synthases [27], GCSs might provide an important target for development of new classes of antifungal agents, especially as their structure(s) and function(s) become better understood. While the IC_{50} for EDO-P4 observed against *A. fumigatus* is $\sim$10 times the concentration found effective in assays with mammalian cells [17], this does not necessarily preclude GCS as a potential target of antifungal therapeutics. The development of PDMP analogs has been directed specifically toward their eventual use in humans for treatment of cancer and GSL storage diseases, and increases in their specificity have been accompanied by a significantly lowered toxicity toward mammalian cells [16,17]. Moreover, despite the sequence similarities between the mammalian and fungal enzymes, there are also significant differences, which suggests that drugs may yet be produced exhibiting much higher specificity for the latter.

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