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Fungal glycolipid hydrolase inhibitors and their effect on *Cryptococcus neoformans*

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Abstract: Pathogenic fungi kill an estimated 1.3 million people each year. This number is predicted to rise as drug resistance spreads, thus antifungal drugs with novel modes of action are urgently required. Fungal endoglycoceramidase-related proteins 1 and 2 (EGCrP-1 and -2), which hydrolyse glucosylceramide and ergosteryl β -glucoside, respectively, are important for fungal cell growth and have been identified as potential targets for drug development. A library of iminosugar derivatives was screened against EGCrP-1 and -2, thereby identifying a number of competitive inhibitors with nanomolar affinities. In addition, a mechanism-based inhibitor was shown to form a covalent derivative with EGCrP-2. Nine of the inhibitors were evaluated against *Cryptococcus neoformans*. Several showed growth inhibitory activity, but only against a *C. neoformans* strain lacking the outer fungal polysaccharide capsule, implying that penetration into the cell is a significant handicap for these inhibitors. Pro-drug versions of these inhibitors may address this issue.

Introduction

Opportunistic fungal pathogens like *Cryptococcus neoformans* kill an estimated 1.3 million people per year, a mortality rate comparable in magnitude to that of tuberculosis and malaria. Fungal infections are especially problematic for immunocompromised patients because they can be difficult to diagnose, leading to late interventions, and are frequently recalcitrant to existing therapies^[1]. Current antifungal agents hail from just a handful of drug classes that target fungal DNA synthesis, membrane and cell wall assembly, or ergosterol production^[2]. These drugs face a number of issues, including narrow species specificity, incompatibilities with other drugs (especially HIV and cancer therapeutics), and toxicities that can outweigh their potential benefit to the infirm^[3]. When treatment fails clinical options are limited. Antimycotics with novel modes of action are urgently required in the clinic.

The glycolipids glucosylceramide (GC) and steryl β -glucoside (SBG) play important roles in cell membrane phenomena, including lipid microdomain and vesicle formation, in both fungi and metazoans^[4]. Perturbation of the biosynthesis and catabolism of these glycolipids in fungi can have pronounced effects on their growth^[5]. Selective targeting of these pathways in fungi over the host represents a promising anti-fungal therapeutic strategy and might be realised by targeting a family of fungal glucosidases dedicated to processing these glycolipids.

Figure 1. (A) GC biosynthesis in the ER of *C. neoformans* involves a quality control mechanism whereby EGCrP-1 degrades immature GCs. (B) EGCrP-2 degrades ergosteryl β -glucoside in the vacuole of *C. neoformans*.

Fungal GC, which is important for pathogenicity and virulence, is quite different from its metazoan counterpart, possessing an extra unsaturation and a branching methyl group on the sphingosine backbone as well as α -hydroxylation on the *N*-acyl group (Figure 1). These unique features of fungal GC are important for its function, so much so that fungi have evolved a quality-control enzyme, EGCrP-1, that degrades 'immature' GC in the ER^[6]. EGCrP-1 is a GH5 β -glucosidase that hydrolyzes

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the glycosidic bond in GC. Disruption of *egcrp1* in *C. neoformans* results in dysregulation of fungal GC biosynthesis and an impaired capsule formation phenotype, implicating EGCrP-1 as a potential drug target^[6]. EGCrP-2 is a vacuolar homolog of EGCrP-1 with a broader substrate specificity profile, displaying the ability to hydrolyze both fungal GC and SBG. Disruption of *egcrp2* in *C. neoformans* led to growth arrest, dysfunctional cell budding and abnormal vacuole morphology, making it an even more promising drug target than EGCrP-1^[7].

Because EGCrP-1 and -2 share sequence homology and a common activity on fungal GC, we reasoned that they could be simultaneously targeted using selective β -glucosidase inhibitors. Their combined inhibition might then lead to the growth arrest or death of fungal pathogens. In order to explore this hypothesis, we conducted a screen against a collection of iminosugar-based competitive β -glucosidase inhibitors (Figure 2) that our laboratory had previously developed as inhibitors of human lysosomal glucocerebrosidase (GCase), an enzyme that has similar activity to EGCrP-1 and -2 on GC^[8]. Azasugars have proved to be potent inhibitors of their cognate glycosidases in a number of cases, with the protonated amine mimicking the oxocarbenium character derived at the reaction transition state^[9]. We were not particularly concerned by off-target inhibition of host GCase, since Gaucher disease patients with greatly diminished GCase activity can survive in health for many years, suggesting that the acute inhibition of host GCase when targeting a fungal infection is not a great concern.

Figure 2. The library of iminosugar-based competitive β -glucosidase inhibitors. EGCrP-1 was screened against compounds 1 to 29. The subset shown within the boxes were screened against EGCrP-2.

Results and Discussion

The enzymes used in our screen were expressed in *Escherichia coli*. Heterologous expression of *C. neoformans* and *A. fumigatus* EGCrP-1 alone or with an N-terminal fusion partner (MBP, GST, NusA) was unsuccessful. Fortunately, EGCrP-1 from *Rhizopus oryzae*, which has high sequence similarity to the *C. neoformans* enzyme, expressed well in *E. coli* with a simple C-terminal hexahistidine tag^[6]. EGCrP-2 of *C. neoformans* expressed well in *E. coli* using a similar approach.

Our recombinant EGCrP-1 possessed no activity on the commercial chromogenic β -glucosidase substrates 4-nitrophenyl β -D-glucopyranoside (PNP-Glc) and 7-methylumbelliferyl β -D-glucopyranoside (MU-Glc), an issue that was overcome by employing the more reactive chromogenic substrate 2,4-dinitrophenyl β -D-glucopyranoside (DNP-Glc), which has a leaving group of pKa 4 versus those of pKa 7 - 8 in the other substrates. Under optimized conditions, the Michaelis-Menten parameters for cleavage of DNP-Glc by EGCrP-1 were determined to be: $k_{cat} = 0.16 \pm 0.01 \text{ s}^{-1}$; $K_m = 2.8 \pm 0.3 \text{ mM}$ and $k_{cat}/K_m = 57 \pm 5 \text{ M}^{-1} \text{ s}^{-1}$. The catalytic efficiency of EGCrP-1 for cleavage of DNP-Glc is approximately 100-fold lower than that with the native substrate, GC ($k_{cat} = (38.3 \pm 0.2) \times 10^{-3} \text{ s}^{-1}$; $K_m = 5.8 \pm 0.3 \text{ uM}$; $k_{cat}/K_m = (6.6 \pm 0.4) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$)^[6]. This level of activity was sufficient for our screen, however a high concentration of enzyme was required to observe rates above the background rate of spontaneous hydrolysis of this highly activated substrate. Despite having a significantly higher affinity for GC over DNP-Glc, the rate constant of EGCrP-1 for cleavage of GC is considerably lower. These rate constants are meagre in comparison to the k_{cat} values of other β -glucosidases, such as *Agrobacterium* β -glucosidase^[10], which cleaves DNP-Glc with a k_{cat} of 88 s^{-1} . This is commensurate with EGCrP-1's role as a quality-control enzyme: a slow turnover of GC is required to prevent the depletion of mature GC while still allowing the hydrolysis of immature forms. On the other hand, EGCrP-2 showed appreciable catalytic activity with both PNP-Glc and DNP-Glc substrates, and hydrolysed DNP-Glc ten-fold faster than did EGCrP-1, with a K_m of $0.46 \pm 0.1 \text{ mM}$, k_{cat} of $29 \pm 2 \text{ s}^{-1}$, and $k_{cat}/K_m = (63 \pm 9) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$.

The library of β -glucosidase inhibitors was screened against EGCrP-1 (Figure 3). The lipophilic iminosugars **10** and **17**

provided > 80% inhibition at a concentration of 1 μM and were selected for further analysis. The K_i values of inhibitors **10** and **17** were thereby determined to be 130 nM and 170 nM, respectively. The screen against EGCrP-2 provided similar hits (Figure 3), despite a number of compounds being omitted from the second screen. The inhibition profile of EGCrP-2 closely mirrors that of EGCrP-1, with inhibitors **16**, **23** and **24** providing more than a 60% reduction of the enzymatic rate and inhibitor **17** showing more than 80% inhibition. Kinetic analysis of the best of these revealed the K_i of inhibitor **17** to be 21 nM when tested against EGCrP-2, eight times more potent than against EGCrP-1.

Figure 3. Percentage inhibition of EGCrP-1 and -2 at inhibitor concentrations of 1.0 μM .

For both enzymes there was a clear trend in their inhibitor sensitivity, with inhibitors becoming more effective with increasing lipophilicity of the iminosugar substituent. One could likely improve the potency of these inhibitors even further by extending the length of the hydrocarbon chain substituent, since the native GC substrate has a longer carbon chain. However, this should be approached with caution as iminosugars with long lipophilic chains tend to be cytotoxic, possibly due to their surfactant-like nature and ability to perturb membranes. Nonetheless, the scaffold of inhibitor **17** has promise, and the lipophilic appendage could be further optimised to enhance potency.^[8]

In an extension to this study, we sought to identify mechanism-based covalent inhibitors of the enzymes, based upon a class of previously identified potent inhibitors of human GCCase¹⁰. In these studies a class of 2-deoxy-2-fluoroglucosides bearing a phosphonoester leaving group was described. The fluorine at C2 destabilises the oxocarbenium ion-like transition states for

formation and hydrolysis of the covalent glycosyl-enzyme intermediate, thus slowing both glycosylation and deglycosylation of the enzyme, while the highly activated leaving group ensures not only that the first glycosylation step is faster than deglycosylation, but also that selectivity for this particular enzyme can be optimized by mimicking, to some extent, the lipophilic ceramide moiety. Inactivation studies were carried out with EGCrP-2 and the most effective of these reagents so far, compound **30** (Figure 4) proved to be effective in this case too, with inactivation parameters of $k_i = 1.11 \text{ min}^{-1}$; $K_i = 11.8 \mu\text{M}$; $k_i/K_i = 94 \text{ mM}^{-1}\text{min}^{-1}$. In fact this inactivator reacted three times more rapidly with EGCrP-2 than it did with human GCCase ($k_i/K_i = 29 \text{ mM}^{-1}\text{min}^{-1}$)^[11]. Interestingly, inactivation of EGCrP-1 was less effective, and did not proceed to completion.

Figure 4. Mechanism-based inactivation of EGCrP-2 by compound **30**. EGCrP-1 was not inactivated by this compound.

Encouraged by these results, inhibitors **10**, **16**, **17**, **20**, **21**, **22**, **23**, **24**, and **30** were tested in vivo against *C. neoformans*. *Cap59*, a mutant strain missing the outer fungal capsule, was used in addition to the wild-type *C. neoformans*. A growth response assay was performed with inhibitors **10** to **24**, incubating the fungi in the presence of 100 μM inhibitor for 72 hours (Figure 5). Compounds **10**, **16** and **17**, the iminosugars with long lipophilic side chains, had noticeable impact on growth in the *cap59* strain with a 40–50% decrease in growth over the course of the assay. Compounds **20** and **21**, possessing shorter side chain appendages, also exhibited some growth inhibition on the *cap59* strain, but to a lesser extent, while **22**, **23** and **24** showed no inhibition. None of the inhibitors afforded a significant decrease in the growth rate of the wild-type strain.

Figure 5. The growth curves of wild-type (KN99a) and acapsular (*cap59*) *C. neoformans* strains over 72 h in YPD media supplemented with EGCrP1/2 inhibitors (100 μ M) at 30°C in the presence of compounds (A) 10, (B) 16, (C) 17, (D) 20, (E) 21, (F) 22, (G) 23 and (H) 24.

A dose response assay was conducted with inhibitors 10, 17 and 30. The results mirrored those seen in the growth response curves: while there was a significant impact on the *cap59* strain, the wild-type strain was largely unaffected even at higher inhibitor concentrations (Figure 6). Surprisingly, the mechanism-based inhibitor 30 showed no activity on the *cap59* or wild-type strains. The lack of significant inhibitory activity in the wild-type strain suggests that the fungal capsule impedes up-take of these compounds.

Figure 6. The sensitivities of wild-type (KN99a) and acapsular (*cap59*) *C. neoformans* strains to EGCrP-1/2 inhibitors 10 and 17. Cultures were incubated with inhibitors for 48 h at 30°C and optical density measured and normalized relative to untreated cultures.

Conclusions

This work sought to evaluate EGCrP-1 and -2 as potential targets for the development of novel antimycotics. Screening against our library of β -glucosidase targeted iminosugar

inhibitors allowed us to identify inhibitors of these enzymes with nanomolar-range inhibition constants. While inhibition of EGCrP-1 and -2 with these chemical tools does appear to slow *C. neoformans* growth, as seen in the activity towards the *cap59* strain, it did not completely arrest fungal growth, even at high compound concentrations. Unfortunately, these compounds were inactive on wild-type *C. neoformans*, suggesting that they may be unable to penetrate the capsule to achieve a sufficient intracellular concentration to inhibit EGCrP-1 and -2. Nonetheless, the bioavailability of these inhibitors may yet be enhanced using traditional 'pro-drugging' strategies. These would serve as very useful chemical probes for studying fungal glycolipid biology.

Experimental Section

Enzyme expression. Codon-harmonized genes encoding *R. oryzae* EGCrP-1 and *C. neoformans* EGCrP-2 were purchased from BioBasic Inc. These genes were ligated into a pET29b expression vector, which was then transformed into electrocompetent BL21*(DE3) *E. coli*. Expression was carried out in LBE-5052 auto-induction media supplemented with 50 μ g mL^{-1} kanamycin at 25 °C for 24 hours. Cells were harvested by centrifugation (17,000 $\times$ g, 20 min, 4 °C) and resuspended in binding buffer (50 mM NaP_i , 500 mM NaCl, 5 mM imidazole, pH 7.5) containing EDTA-free protease inhibitor cocktail and lysozyme (0.1 mg mL^{-1}) by incubating at 4 °C for 30 min. Benzonase (1 μ L) was added to the mixture then lysis was effected by sonication. The lysate was centrifuged (17,000 $\times$ g, 20 min, 4 °C) and the supernatant collected. The supernatant was filtered (0.45 μ m) and then subjected to immobilized metal-ion affinity chromatography (elution buffer: 50 mM NaP_i , 500 mM NaCl, 500 mM imidazole, pH 7.5). The protein obtained was estimated to be >95% pure by Coomassie-stained SDS-PAGE. Protein concentration was determined by Bradford assay. Recombinant expression of soluble hexahistidine-tagged RoEGCrP-1 was achieved in high yield of >50 mg L^{-1} . Recombinant expression of soluble hexahistidine-tagged EGCrP-2 was achieved in an average yield of 20 mg L^{-1} .

Chemical synthesis. Full details of the synthesis and characterisation of the library of azasugars were published previously¹⁰. Benzyl benzyl-(2-deoxy-2-fluoro- β -D-glucopyranosyl) phosphonate (30) was synthesized according to protocols previously reported.^[11] Briefly, acetylated 2-deoxy-2-fluoroglucopyranosyl bromide was coupled to the corresponding benzyl phosphonic acid under Koenigs-Knorr conditions promoted by silver carbonate. The resulting product was subsequently de-O-acetylated with catalytic sodium methoxide to yield compound 30 as a 1:1 mixture of epimers at the phosphorus centre.

Inhibitor screen. A Michaelis-Menten plot was constructed and k_{cat} and K_m values were determined for the hydrolysis of 2,4-dinitrophenyl glucoside by EGCrP-1 and -2. Reaction rates were measured by monitoring the net change in absorbance at 400 nm. The inhibitor screen was carried out in a Synergy HI plate reader. For analysis of RoEGCrP-1, 100 nM of enzyme, 3 mM DNPGLc (K_m), and 1 μ M of inhibitor were used in each reaction. The screen was run in duplicate and results were verified on a Cary 300 spectrophotometer. In the screen against CnEGCrP-2, 100 nM of enzyme, 0.4 mM DNPGLc (K_m), and 1 μ M of inhibitor were used in each reaction. The screen was run in duplicate. Compounds that resulted in >80% inhibition were selected for K_i determination. K_i determinations were performed using two to four different substrate concentrations. For each inhibitor, a range of four to six inhibitor concentrations bracketing the K_i value was used for each substrate concentration. Dixon plots (1/v vs [I]) were constructed to

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validate the use of a competitive inhibition model and to assess the fit of the rest of the data. A competitive inhibition model was fit to the data by using non-linear regression analysis with GraFit 7.0.

Time-dependent inhibition. The kinetic parameters for the inactivation of EGCrP-2 were determined as follows: samples of the enzyme were incubated in buffer in the presence of a range of concentrations of the inhibitor. Aliquots (20 µl) of these inactivation mixtures were removed at time intervals and diluted into assay cells containing a larger volume (180 µl) of substrate (at saturating concentrations). This effectively stops the inactivation both by dilution of the inactivator and by competition with an excess of substrate. The residual enzymatic activity was determined from the rate of hydrolysis of the substrate, which is directly proportional to the amount of active enzyme. The process was monitored until the enzyme was 80–90% inactivated. Pseudo-first order rate constants (k_{obs}) for each inactivator concentration were calculated by fitting plots of the residual activity versus time to a single exponential equation using GraFit 7.0. These values of k_{obs} were then fit to the equation below describing the inactivation process to obtain values of k_i and K_i :

$$k_{\text{obs}} = k_i[I] / (K_i + [I])$$

C. neoformans growth response assay. Two *C. neoformans* strains, wild-type (KN99a) and the acapsular mutant strain *cap59*, were assessed for their sensitivity to the EGCrP-1 and -2 inhibitors. To perform the assays, the fungal cells were seeded in micro-titre wells in YNB media at 1×10^6 cells/ml (–0.08 optical density at 600 nm [OD₆₀₀]) in triplicate with or without 100 µM inhibitor and incubated at 30°C. For each strain, the OD₆₀₀ was measured every 2 h after initiation of the experiments for 72 h.

C. neoformans dose response assay. The dose response of two *C. neoformans* strains, wild-type (KN99a) and the acapsular mutant strain *cap59*, to compounds **10** and **17** was assessed. To perform the assays, the fungal cells were seeded in micro-titre wells in YNB media at 1×10^6 cells/ml (OD₆₀₀ ≈ 0.08) in triplicate with or without inhibitor and incubated for 48 h at 30°C. For each concentration of the drug and for each strain, the increase in OD₆₀₀ for treated samples was normalized against the untreated control to determine the percentage growth inhibition.

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FULL PAPER

A library of iminosugar derivatives was tested against fungal enzymes EGCrP-1 and 2 allowing for the identification of nanomolar-range inhibitors. Several inhibitors were tested against *Cryptococcus neoformans* and showed growth inhibitory activity towards the acapsular strain.

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