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Novel Antifungal Drug Discovery Based on Targeting Pathways Regulating the Fungus-Conserved Upc2 Transcription Factor

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Infections by *Candida albicans* and related fungal pathogens pose a serious health problem for immunocompromised patients. Azole drugs, the most common agents used to combat infections, target the sterol biosynthetic pathway. Adaptation to azole therapy develops as drug-stressed cells compensate by upregulating several genes in the pathway, a process mediated in part by the Upc2 transcription factor. We have implemented a cell-based high-throughput screen to identify small-molecule inhibitors of Upc2-dependent induction of sterol gene expression in response to azole drug treatment. The assay is designed to identify not only Upc2 DNA binding inhibitors but also compounds impeding the activation of gene expression by Upc2. An AlphaScreen assay was developed to determine whether the compounds identified interact directly with Upc2 and inhibit DNA binding. Three compounds identified by the cell-based assay inhibited Upc2 protein level and *UPC2-LacZ* gene expression in response to a block in sterol biosynthesis. The compounds were growth inhibitory and attenuated antifungal-induced sterol gene expression *in vivo*. They did so by reducing the level of Upc2 protein and Upc2 DNA binding in the presence of drug. The mechanism by which the compounds restrict Upc2 DNA binding is not through a direct interaction, as demonstrated by a lack of DNA binding inhibitory activity using the AlphaScreen assay. Rather, they likely inhibit a novel pathway activating Upc2 in response to a block in sterol biosynthesis. We suggest that the compounds identified represent potential precursors for the synthesis of novel antifungal drugs.

Candida species cause systemic infections in the immunocompromised, such as HIV patients, those receiving cancer chemotherapy, and organ transplant patients (1–5). Systemic fungal infections are a mortality factor for individuals with HIV (4), and affect the quality of life of elderly diabetics (6, 7). The fungal sterol biosynthetic pathway is the target for many of the commonly administered antifungal drugs (see Fig. 1A) (8–11). There are several classes of sterol pathway targeting drugs, the most common being the azoles, which includes the N-substituted imidazoles fluconazole and miconazole and the new triazole derivatives itraconazole and posaconazole (12). In addition, there are allylamines (terbinafine) (13) and morpholine classes of drugs (fenpropimorph) (14), as well as sterol binders (amphotericin B and nystatin) (15). All are intended to reduce sterol levels or sequester sterol. Finally, there are the echinocandins that inhibit β-glucan synthase, which blocks cell wall biosynthesis (16, 17).

Saccharomyces cerevisiae, *Candida albicans*, and *Candida glabrata* produce ergosterol as the end product of sterol biosynthesis rather than cholesterol (18). The ergosterol biosynthetic pathway is regulated under various growth conditions by multiple transcription factors (TFs) (19–24). Upc2 is a member of the fungus-conserved Zn_2-Cys_6 binuclear cluster TFs (25). There is a homolog of *S. cerevisiae* Upc2 (ScUpc2), ScEcm22 (26, 27), a single *Candida albicans* ortholog, *C. albicans* Upc2 (CaUpc2) (28), and two *C. glabrata* Upc2 (CgUpc2) orthologous isoforms in *C. glabrata* (29). Upc2 induces ergosterol biosynthetic gene expression in response to azole antifungal drug treatment (30). It does so by binding to yeast sterol response elements (SREs) in the promoters of sterol genes (26, 30–35). Strains lacking Upc2 and Ecm22 are sensitive to antifungal treatment and are themselves growth compromised (28). Clinical isolates with gain-of-function mutations in *CaUPC2* are resistant to azole therapies (35–38).

There are multiple reviews discussing the feasibility of HTS (high-throughput screen) identification of compounds directly interacting with TFs and their therapeutic use (39–42). Although this area has met with limited success, many of the compounds isolated show surprising selectivity, and in some cases *in vivo* efficacy (43–46). The Upc2 transcription factor is a critical regulator of the antifungal drug response, and as such represents an excellent target for antifungal drug discovery. Antifungal resistance requires induced sterol gene expression. Upc2 inhibition would eliminate the most downstream event required for resistance, thus circumventing a number of present resistant mechanisms.

We have developed two independent high-throughput screens aimed at identifying small-molecule Upc2 inhibitors. One is a homogenous whole-cell assay that screens for compounds reducing fluconazole-induced *UPC2-LacZ* gene expression. The second is an *in vitro* AlphaScreen assay (PerkinElmer) designed to determine whether compounds directly or indirectly inhibit Upc2 DNA binding. Our efforts have resulted in the identification of three small-molecule compounds inhibiting Upc2-dependent transcriptional signaling *in vivo*, while also reducing the level of Upc2 that is observed in response to a block in sterol biosynthesis (47).

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MATERIALS AND METHODS

Yeast strains and miscellaneous methods. *Saccharomyces cerevisiae* strains were constructed in the W303 strain background (*MAT***a** *ura3-52 leu2 his3 lys2 ade2*). The *Candida albicans* strain used was BWP17 (*ura3/ura3 arg4/arg4 his1/his1*). The wild-type *Candida glabrata* strain is 66032 (American Tissue Culture Collection). Strains were grown in YEPD (1% yeast extract, 2% Bacto peptone, 2% glucose) or in synthetic minimal medium (0.67% yeast nitrogen base [Difco]) supplemented with the appropriate amino acids and adenine. For the screen, fluconazole was added directly to liquid media. Yeast transformation was performed using the procedure described by Ito et al. (48). For routine propagation of plasmids, *Escherichia coli* XL1-Blue cells were used and grown in LB medium supplemented with ampicillin (150 μg/ml). Fluconazole and dimethyl sulfoxide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Microtiter plates were purchased from Greiner Bio-One (Monroe, NC, USA), and cell strainers (70 μm) were purchased from Becton, Dickinson (Franklin Lakes, NJ, USA). VB00075177 (compound 1), VB00075853 (compound 2), and VB00075845 (compound 3) were purchased from TimTec for rescreening.

Plasmid constructions for HTS. The yeast integrating plasmid, YIp353, was used to construct all plasmids. YIp353-*UPC2-LacZ* contains a wild-type *ScUPC2-LacZ* promoter fusion containing two SREs that ScUpc2 binds to in order to regulate fluconazole-induced transcription (67). Seven hundred base pairs of the *ScUPC2* promoter drive the expression of β-galactosidase (49). YIp353-$^{sre-}$*UPC2-LacZ* contains a *ScUPC2* promoter deleted for both SREs, which were deleted by site-directed mutagenesis. The plasmid YIp353-*PGK1-LacZ* contains the wild-type *ScPGK1* (phosphoglycerate kinase 1) promoter. Site-directed mutagenesis was performed using the QuikChange Multi site-directed mutagenesis kit (Stratagene, La Jolla, CA), and mutations were verified by DNA sequencing. YIp353 constructs were integrated at the endogenous *URA3* locus by digestion with StuI and transformation into a wild-type *S. cerevisiae* strain (YJN16).

Compound libraries. Ten small-molecule compound libraries were purchased from commercial sources. FDA-approved drug libraries included the Prestwick Collection (Prestwick Chemical, Illkirch, France; 1,120 compounds) and the Enzo FDA-approved compound library (Enzo Life Sciences, Plymouth Meeting, PA, USA; 640 compounds). The Natural Products Library was purchased from TimTec (Newark, DE, USA; 640 compounds). The kinase-targeted library Acti-Targ-K (960 compounds) was obtained from TimTec. Diversity compound sets were as follows: Maybridge Micro HitFinder (Thermo Fisher Scientific, Waltham, MA, USA; 14,400 compounds), Chembridge DIVERSet (ChemBridge, San Diego, CA, USA; 10,000 compounds), TimTec Diversity Set and ActiProbe 25 (TimTec; 10,000 and 25,000 compounds, respectively), and 15,280 compounds from ChemDiv (San Diego, CA, USA). Compounds were screened at a concentration of 5 to 10 μM.

Ultrahigh-throughput screen (uHTS). *S. cerevisiae* strain YJN3672 (W303 *ura3*::*URA3*::*UPC2-LacZ*) was grown to log phase (optical density at 600 nm [OD_{600}] of 0.8 to 1.2) in minimal yeast medium lacking uracil. Cells were diluted 1:20 into the final assay volume of 4 μl. Test compounds, dried in wells of solid white 1,536-well microtiter plates, were redissolved for 30 min at 30°C in 2 μl/well of a 1:10 dilution of log-phase cells. Fluconazole, diluted from a 60-mg/ml dimethyl sulfoxide stock into culture medium, was then added to a concentration of 35 μg/ml, in a final assay volume of 4 μl, and incubation was continued for 4 h at 30°C. A fluconazole concentration of 35 μg/ml was used to stimulate the activity of cells at a concentration where sterol-responsive activity was at 80% of maximum. A cutoff level of 50% of control was used for choosing compounds inhibiting Upc2-dependent β-galactosidase activity, at a screening concentration of 8 μM. β-Galactosidase detection was performed using BetaGlo (Promega, Madison, WI, USA). After 30 min at room temperature, luminescence was detected in the EnVision multimode plate reader (PerkinElmer, Waltham, MA, USA). Curve fitting was performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA) or ActivityBase software (IDBS, Guildford, United Kingdom).

Mammalian cell toxicity assay. HEK293 cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, sodium bicarbonate, penicillin, and streptomycin and were grown in 5% CO_2 at 37°C. The cells were seeded at 2×10^5 cells/ml in 4 μl of medium and test compound in solid white 1,536-well microtiter plates (Greiner Bio One, Monroe, NC). The plates were incubated for 48 h. The plates were equilibrated to room temperature before using Cell Titer-Glo luminescent cell viability assay according to the manufacturer's directions (Promega, USA). Luminescence was detected using the EnVision 2103 multilabel reader (PerkinElmer, USA).

Expression and purification of GST-Upc2 DNA binding domain fusion proteins. The *UPC2* gene fragments encoding the DNA binding domains from *Candida albicans*, *S. cerevisiae*, and *C. glabrata* were amplified by PCR using chromosomal DNA as a template. The PCR products were ligated into the *E. coli* expression vector pGEX-4T3, using BamHI and EcoRI, to create pGEX-*CaUPC2*, pGEX-*ScUPC2*, and pGEX-*CgUPC2*. All sequences were fused in frame to vector-derived sequences encoding glutathione *S*-transferase (GST), followed by a stop codon. DNA sequencing was performed to confirm that no mutations were introduced into the sequences, and the vectors were transformed into *E. coli* strain BL21 for expression of the fusion proteins.

To induce expression of the fusion proteins, transformed *E. coli* BL21 cells were grown to mid-log phase (A_{600} of ~0.5) at 37°C in Luria broth containing 100 μg/ml ampicillin and 150 μM $ZnSO_4$. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and cultures were incubated with shaking at 37°C for an additional 4 h. Soluble cytoplasmic protein was extracted from cells using BugBuster master mix (EMD Chemicals, Gibbstown, NJ) according to the manufacturer's instructions, except that the BugBuster was supplemented with 10 μM $ZnSO_4$. GST fusion proteins were purified using a GSTrap column (GE Healthcare, Parsippany, NJ), following the manufacturer's instructions, except that all buffers contained 10 μM $ZnSO_4$.

AlphaScreen DNA binding assay. AlphaScreen is a bead-based binding assay technology. Donor beads contain a photosensitizer that converts ambient oxygen to a short-lived form of singlet oxygen upon illumination at 680 nm. Singlet oxygen diffuses up to 200 nm in solution before it decays. Interaction between GST-CaUpc2 and SRE brings the donor beads into close proximity with the acceptor beads, allowing singlet oxygen to activate a thioxene derivative in the acceptor beads, leading to the emission of light between 520 and 620 nm. In the absence of acceptor beads or in the presence of acceptor beads and a small molecule inhibiting DNA binding, the singlet oxygen decays to the ground state with no light emission. Donor beads can release up to 60,000 singlet oxygen molecules per second, resulting in signal amplification. Because signal detection is performed in a time-resolved manner and at a lower wavelength than excitation, interference from fluorescent test compounds is low.

Binding of the CaUpc2 DNA binding domain to DNA was detected using streptavidin-coated AlphaScreen donor beads (PerkinElmer, Waltham, MA), which capture biotinylated DNA, and anti-GST-coated AlphaScreen acceptor beads, which bind to the GST-CaUpc2 fusion protein. The DNA probe sequence used for the screening assay was: *SRE*, 5′-biotin-TEG-CTGTATTG**TCGTATA**AAAGTGG-3′ and 5′-CCACTTT**TATACGA**CAATACAG-3′, where TEG is a triethylene glycol spacer (*C. albicans ERG2* consensus). The probe was hybridized to form a biotinylated, double-stranded oligonucleotide by combining them at a concentration of 50 μM each in a buffer consisting of 100 mM potassium acetate (KOAc) and 30 mM HEPES (pH 7.4). The mixture was heated to 91 to 95°C for 2 min and allowed to equilibrate to 25°C.

For this assay, fusion protein was diluted to 8 nM (0.34 μg/ml) in an assay buffer consisting of 25 mM HEPES (pH 7.0), 200 nM NaCl, 0.1% Tween 20, and 3 μM $ZnSO_4$. Protein was then combined with an equal volume of acceptor beads, which had been diluted to 80 μg/ml in assay buffer, and 4 μl/well of the mixture was transferred to a white, 1,536-well

assay plate. The plate was incubated for 30 min at room temperature. Biotinylated, double-stranded DNA was diluted to 40 nM in assay buffer and combined with an equal volume of 80 μg/ml donor beads in assay buffer. Four microliters of this mixture was added to each well of the assay plate, and the plate was incubated for an additional hour at room temperature in the dark. The signal was detected using a PerkinElmer EnVision microplate reader.

IC_{50} determination. Yeast cultures were diluted to 5×10^3 cells per ml in YEPD medium. Cells (100 μl) were distributed to wells of a 96-well flat-bottom plate, except for row A, which received 200 μl. Drug was added to row A at the desired concentration and then serially 2-fold diluted to rows B through F; row G served as a drug-free control, and row H served as a cell free control. The plates were incubated at 35°C for 24 h. Absorbance at 620 nm was read with a microplate reader (FluorStar Galaxy, BMG); background due to medium was subtracted from all readings. The IC_{50} was defined as the lowest concentration inhibiting growth at least 50% relative to the drug-free control.

Chromatin immunoprecipitation (ChIP) assays. Yeast cells from a single colony were grown in YEPD liquid medium at 30°C overnight. Exponentially growing mother cultures were back-diluted to 5×10^5 cells/ml for *S. cerevisiae* cells and 1×10^5 cells/ml for *C. albicans* or *C. glabrata* cells. Each compound was individually added to diluted cultures at IC_{50} levels, and cells were incubated for 30 min at 30°C. Fluconazole at 50 μg/ml was added directly to cultures and incubated for additional 6 h at 30°C. Compounds and fluconazole were dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemicals).

After incubation with fluconazole, cells were treated with 1% formaldehyde for 2 h at room temperature. Cross-linking was stopped by the addition of 1× TBS (Tris-buffered saline) (1× TBS is 20 mM Tris-HCl [pH 7.6] and 150 mM NaCl). The cells were pelleted, washed with 1× TBS, and again pelleted. The cells were then lysed using lysis buffer (50 mM HEPES [pH 7.5], 140 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1% sodium deoxycholate, and a protease cocktail) and cold glass beads and spun down to remove cellular debris. Two hundred fifty milligrams of the resulting supernatant was used for immunoprecipitating ScUpc2-MYC-bound to each SRE for 1 day at 4°C, using 20 μl of anti-MYC monoclonal antibody. Immunoprecipitated ScUpc2-SRE complexes were isolated after 1 h at 4°C using protein G Sepharose/agarose beads. The beads were washed with lysis buffer containing 500 mM NaCl, and finally with a solution of 10 mM Tris-HCl (pH 8.0), 0.25 M LiCl, 0.5% NP-40, and 0.5% sodium deoxycholate. ScUpc2-DNA complexes were eluted from beads using a solution of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1% SDS. Cross-linking was reversed using 5 M NaCl at 65°C for 6 h. Samples were then diluted with a solution of 500 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.67% SDS. Samples were treated with 250 μg proteinase K for 1 h at 37°C. DNA was eluted from samples using the DNeasy kit (Qiagen). Purified DNA and PCR amplification were used to quantitate the concentration of ScUpc2-MYC to each SRE.

Quantitative reverse transcription-PCR (qRT-PCR) analyses. Cells were grown to exponential log phase at 30°C. Total RNA was isolated as described previously (50). Total RNA was resuspended in diethyl pyrocarbonate (DEPC)-treated water. Fifty nanograms of RNA, 11.5 μl of the SYBR green master mix (Quanta), and 5 μM primer sets were loaded in triplicate onto a 96-well plate. RT-PCR amplification and analysis were completed using the Stratagene Max-Pro (Mx3000P) software version 4.0.

Western blot analysis. Yeast strains were grown at 30°C in Ura^- medium (medium lacking uracil) to exponential log phase. Cells were treated with compound alone or treated with compound in the presence of fluconazole for 16 h. Cell extracts were then isolated, and the ScUpc2-MYC level was detected using Western blot analysis. Briefly, cells were disrupted with cold glass beads and lysis buffer [Tris base (pH 7.9) containing $(NH_4)_2SO_4$, $MgSO_4$, glycerol, and EDTA]. Lysed cells were centrifuged at 3,300 rpm for 5 min at 4°C to obtain a total cell extract. Thirty micrograms of total cell extract was resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. Western blot analysis was performed using anti-MYC and anti-GAPDH (antibody against glyceraldehyde-3-phosphate dehydrogenase) antibodies at a 1:1,000 dilution in TBS plus 1% nonfat milk. Secondary antibodies were horseradish peroxidase-conjugated IgG secondary antibodies diluted to 1:2,000 in TBS plus 1% nonfat milk.

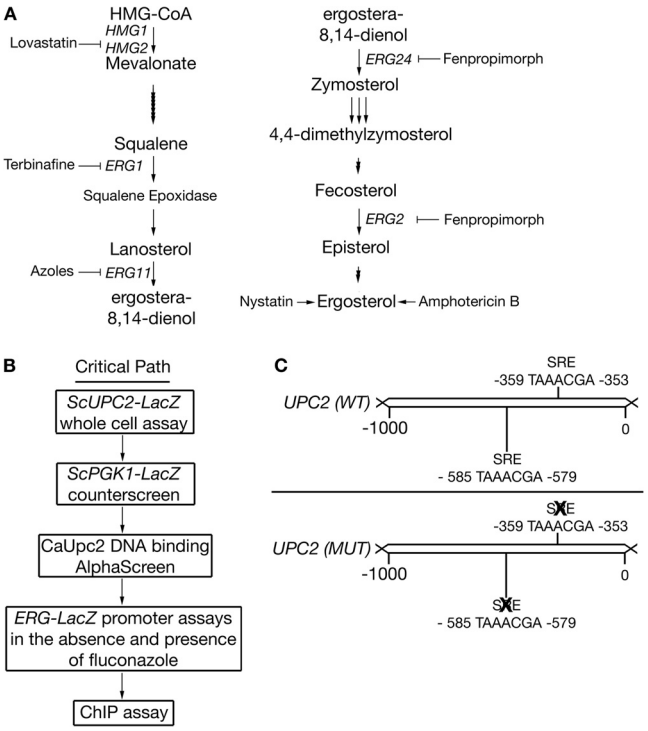

FIG 1 (A) Schematic depicting the yeast ergosterol pathway. The arrows represent individual enzymatic steps in the biosynthetic pathway. Steps inhibited by an antifungal compound are indicated. (B) Schematic of the critical path used for identifying compound inhibitors of Upc2. All compounds isolated that were positive in the *UPC-LacZ* assay for inhibition were tested in the *PGK1-LacZ* assay. The compounds that had positive results in the whole-cell assay that did not inhibit *PGK1-LacZ* activity were screened in the AlphaScreen assay. Compounds carried forward were assayed for inhibiting Upc2-dependent transcription factor activity. Positive compounds remaining were tested for inhibitory synergy with fluconazole. (C) Schematic of the *UPC2* promoter indicating the location of each SRE. WT, wild type; MUT, mutant.

RESULTS

A whole-cell HTS designed to identify compounds inhibiting ScUpc2-dependent transcription. A high-throughput screening campaign was undertaken, which was designed to identify small-molecule compounds targeting the Upc2 transcription factor. The budding yeast *Saccharomyces cerevisiae* was used as a model. *S. cerevisiae* is more amenable to genetic manipulation than *C. albicans* or *C. glabrata* is, which helped in engineering a strain suitable for a high-throughput cell-based assay (51). We are aware that a potential limitation in using the *S. cerevisiae LacZ* strain for screening, rather than a *C. albicans LacZ* strain, is that compounds directly inhibiting only CaUpc2-SRE binding may not be identified. However, based on the k_m values determined using *in vitro* AlphaScreen assays (see below), it was believed the compounds identified would inhibit ScUpc2 and CaUpc2 DNA binding (Fig. 1B).

An *S. cerevisiae* strain was constructed containing an integrated copy of a *ScUPC2-LacZ* promoter fusion (Fig. 1C). The goal of the

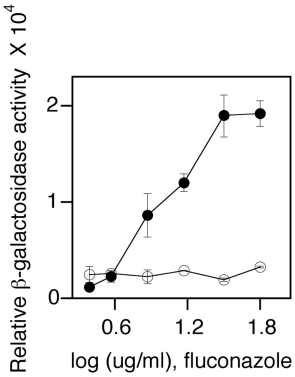

FIG 2 Cells expressing an *UPC2-LacZ* promoter fusion show a dose-dependent increase in β-galactosidase activity in response to increasing concentrations of fluconazole (in micrograms per milliliter). Cells were diluted 1:20 into the final assay volume of 4 μl. Test compounds in wells of solid white 1,536-well microtiter plates were redissolved in medium containing cells. Increasing concentrations of fluconazole were then added in a final assay volume of 4 μl, and cells were incubated for 4 h at 30°C. β-Galactosidase activity was performed using BetaGlo (Promega, Madison, WI, USA). β-Galactosidase activity of the *UPC2-LacZ* wild-type strain (solid black circles) and *ecm22 upc2 UPC2-LacZ* strain (white circles) are shown.

whole-cell screen was to identify compounds reducing fluconazole-induced *ScUPC2-LacZ* promoter activity. A second strain was constructed; this strain carried an integrated copy of an *ScPGK1-LacZ* promoter fusion lacking SREs. This strain was used in a counterscreen designed to eliminate compounds inhibiting general RNA polymerase II transcription (Fig. 1B).

The fluconazole concentration to be used in the whole-cell assay was first determined. Cells were assayed for *ScUPC2-LacZ*-dependent β-galactosidase activity in the presence of various concentrations of fluconazole. β-Galactosidase activity showed a dose dependence on the concentration of fluconazole present (Fig. 2, black circles). An *Scupc2 Scecm22 ScUPC2-LacZ* null strain had no observable fluconazole-induced SRE activity, as both the ScUpc2 and ScEcm22 transcription factors that bind to SREs were absent (not shown). A wild-type strain harboring a *ScUPC2-LacZ* promoter where the SREs were mutated (YIp353-$^{sre-}$*ScUPC2-LacZ*) lacked β-galactosidase activity (Fig. 2, white circles). Thus, any fluconazole-dependent activity observed was SRE dependent and required ScUpc2 for activity.

The commercially available Prestwick small-compound collection was initially screened (52). The Prestwick collection contains 1,200 FDA-approved small-molecule compounds. Z' values for the whole-cell assay were found to be between 0.6 and 0.8. A Z' calculation quantifies the suitability of an assay for use in a high-throughput screen. A Z' value above 0.5 indicates an accuracy sufficient for screening.

Not unexpectedly, a number of compounds isolated enhanced fluconazole-dependent *UPC2-LacZ* promoter activity. Many were representatives of the azole, statin, and allylamine families. Identifying synergistic activators indicates that the whole-cell assay is a valid method to identify Upc2 transcription factor inhibitors. Eleven Prestwick compounds that inhibited fluconazole-induced *ScUPC2-LacZ* activity were isolated; IC_{50}s were between ~3.0 and 20 μM, and in all but one case were >50 μM for *ScPGK1-LacZ* promoter activity. The 11 compounds included those with antifungal activities, and several nonsteroidal anti-inflammatory drugs (NSAIDs) and steroid molecules.

TABLE 1 Hits from whole-cell uHTS screen using the Venenum 76,000 diversity small compound collection

Compound	IC_{50} (μM) on the following promoter activity:		AlphaScreen assay % control at 30 μM
	ScUPC2-LacZ ($n = 4$)	*ScPGK1-LacZ* ($n = 2$)	
VB00075177	0.29 ± 0.07	No inhibition at 30 μM	99 ± 10
VB00075853	0.63 ± 0.06	>20 μM	51 ± 5
VB00075845	0.92 ± 0.10	>20 μM	51 ± 3
VB00049027	1.5 ± 1.0	No inhibition at 60 μM	83 ± 7
VB00008679	2.5 ± 0.5	No inhibition at 60 μM	90 ± 10
VB00073293	2.6 ± 0.7	No inhibition at 60 μM	94 ± 12
VB00059166	3.3 ± 0.5	No inhibition at 60 μM	84 ± 6
VB00068594	4.2 ± 0.7	No inhibition at 60 μM	69 ± 3

A 76,000-compound diversity set collection was next screened (see Materials and Methods). The eight compounds identified inhibited fluconazole-induced *ScUPC2-LacZ* promoter activity; IC_{50}s were between 0.3 and 4 μM (Table 1), while IC_{50}s for the *PGK1-LacZ* promoter were between 20 and 60 μM. Molecular structures are shown in Fig. 3. Among the eight compounds, compounds 1, 2, and 3 were closely related. Several other analogs containing the same 2-phenyl-3-nitro-2H-chromene core showed similar activity. However, not all 3-nitro-2H-chromene compounds were active, indicating that the core was not solely responsible for activity.

AlphaScreen assay designed to validate compounds as direct Upc2 DNA binding inhibitors. A whole-cell reporter assay cannot distinguish between direct and indirect inhibition of DNA

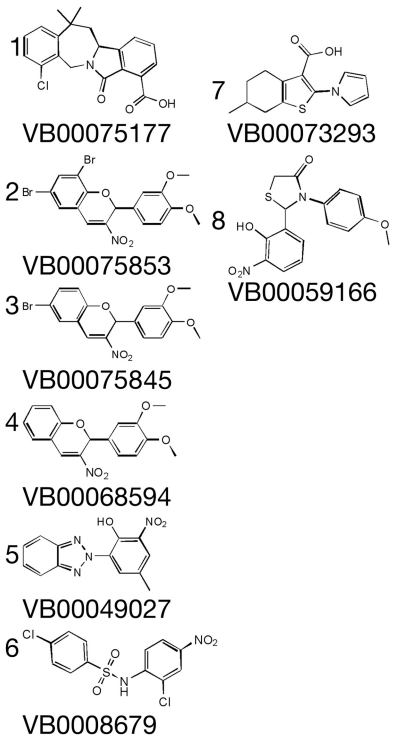

FIG 3 Molecular structures of the identified compounds The molecular structures and VB numbers of compounds 1 to 8 are shown. The compounds were isolated using the commercial small-molecule collection.

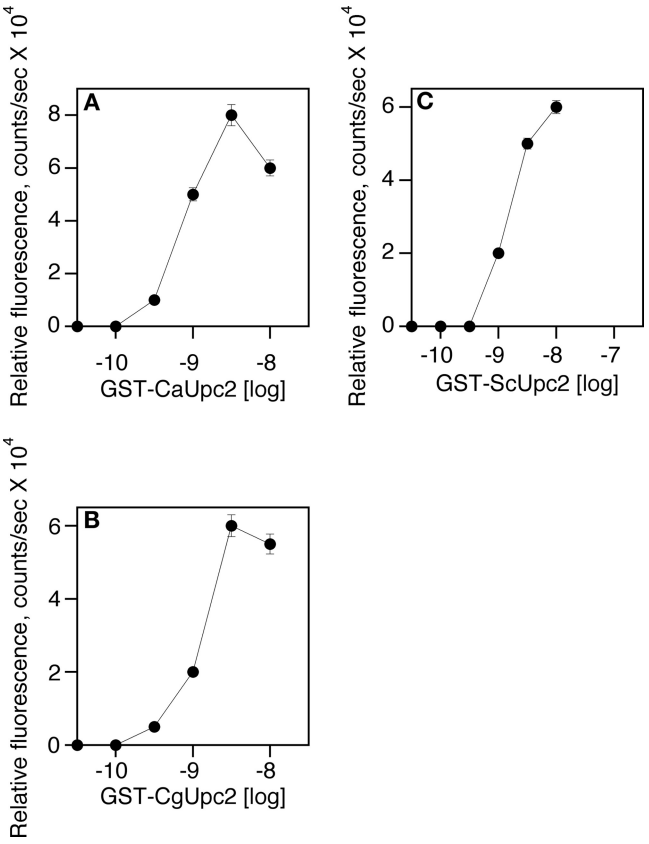

FIG 4 Upc2 DNA binding in the AlphaScreen assay shows a dose-dependent increase in activity in response to an increasing concentration of protein. Increasing concentrations of fusion proteins were diluted in assay buffer. Protein was combined with an equal volume of acceptor beads, which had been diluted in assay buffer. Part of the mixture (4 μl/well) was transferred to a white, 1,536-well assay plate. The plate was incubated for 30 min at room temperature. Biotinylated, double-stranded DNA was diluted in assay buffer and combined with an equal volume of donor beads in assay buffer. Four microliters of this mixture was added to each well, and the plate was incubated for an additional hour at room temperature in the dark. The signal was detected using a PerkinElmer EnVision microplate reader. GST-CaUpc2, glutathione *S*-transferase (GST) fused to the *C. albicans* Upc2 DNA binding domain; GST-ScUpc2, *S. cerevisiae* Upc2 DNA binding domain; GST-CgUpc2, *C. glabrata* Upc2 DNA binding domain.

binding. Thus, an *in vitro* AlphaScreen assay that detects the direct interaction of *C. albicans* CaUpc2 with its SRE was developed. The assay was set up as described in Materials and Methods.

The CaUpc2 concentration that would be used in the assay was determined. The binding domain of CaUpc2 (amino acids [aa] 1 to 170) was used and purified as a GST fusion protein. Binding to the consensus SRE from *CaERG2* was determined (31). An apparent K_m of 50 nM was calculated for CaUpc2 (Fig. 4A). Similar K_m values were obtained for the DNA binding domains of ScUpc2 and CgUpc2 (Fig. 4B and C).

Determining Upc2 DNA binding inhibitory activity. The 11 Prestwick collection hits from the *ScUPC2-LacZ* assay were tested. None inhibited DNA binding, indicating their direct target(s) is not CaUpc2. The entire Prestwick collection was screened and resulted in the identification of 11 compounds. These compounds were given low priority as precursors for a hit-to-lead program based on their molecular structures and mode of action.

The eight hits identified from the *ScUPC2-LacZ* commercial library screen were tested. Each compound did not inhibit CaUpc2 DNA binding. None harbored activity against the *ScPGK-LacZ* promoter, and they were not cytotoxic using a HEK293 cell proliferation assay. Based on this data, the compounds identified are most likely regulating a signaling pathway required for Upc2 activation, rather than directly inhibiting Upc2 DNA binding.

On the basis of the fact that all compounds tested did not inhibit DNA binding, we chose to carry forward three compounds showing the lowest IC_{50}s in the *UPC2-LacZ* cell assay (VB00075177, VB00075853, and VB00075845, referred to as compounds 1, 2, and 3, respectively). The compounds were tested for the ability to inhibit *Scecm22* cell growth; these cells contain only ScUpc2. The three compounds inhibited cell growth at low micromolar concentrations. The IC_{50}s were determined for the three compounds and were 1.2 μM ± 0.3 μM for compound 1, 2.5 μM ± 0.2 μM for compound 2, and 0.3 μM ± 0.1 μM for compound 3. These compounds inhibited wild-type *S. cerevisiae* and *C. glabrata* strains with similar IC_{50}s but had no effect on wild-type *C. albicans* cell growth.

Each compound blocks fluconazole-induced *UPC2* promoter activity. Compounds were tested for the ability to inhibit ScUpc2-dependent transcription in the presence of fluconazole. The expression of *ScERG11* and *ScERG25* is induced in the presence of fluconazole through Upc2 activation (53–55). *ScUPC2* expression is also induced (47), while *ScPGK1* expression remains at basal level. As expected, *Scecm22* null cells grown in the presence of fluconazole alone induced the expression of *ScUPC2*/*ERG11*/*ERG25* (~2- to 4-fold) (Fig. 5A to C, without FLC versus with FLC). The expression level of *ScPGK1-LacZ* did not change (Fig. 5D). The addition of each compound abolished fluconazole-induced *ScERG25*/*ScERG11*/*ScUPC2* expression, while having no effect on *ScPKG1-LacZ* basal expression.

Compounds were tested for inhibiting fluconazole-induced gene expression in the pathogenic fungi, *C. albicans* and *C. glabrata*. No effects on *C. albicans* expression were seen in the presence of each compound and fluconazole. The addition of each compound to *C. glabrata* cells attenuated fluconazole-induced expression (Fig. 6A to D). Thus, the three compounds inhibit sterol gene expression in *S. cerevisiae* and *C. glabrata*.

Fluconazole-dependent ScUpc2 protein expression is decreased in the presence of compound. ScUpc2 protein level was determined to see whether loss of *ScUPC2* transcription resulted in loss of protein. An increase in ScUpc2-MYC protein expression was seen in the presence of fluconazole (Fig. 7, con versus FLC) that was eliminated by addition of each compound (Fig. 7, con versus FLC + 1, FLC + 2, or FLC + 3).

Each compound reduces ScUpc2 binding to endogenous SRE sites. The level of *in vivo* ScUpc2 SRE binding was determined in the presence of each compound. The *ScUPC2* promoter contains two SREs (SRE1, positions −585 to −379; SRE2, positions 359 to 353). ChIP was used to determine the level of ScUpc2 binding to each SRE. A low level of bound ScUpc2 was seen at both SREs in the absence of fluconazole (Fig. 8). Upon the addition of fluconazole, a 3.5-fold increase in binding was seen to SRE2 (Fig. 8, bottom panel). No fold increase over basal binding was seen for SRE1 (Fig. 8, top panel). Each compound abolished the increase seen for SRE2.

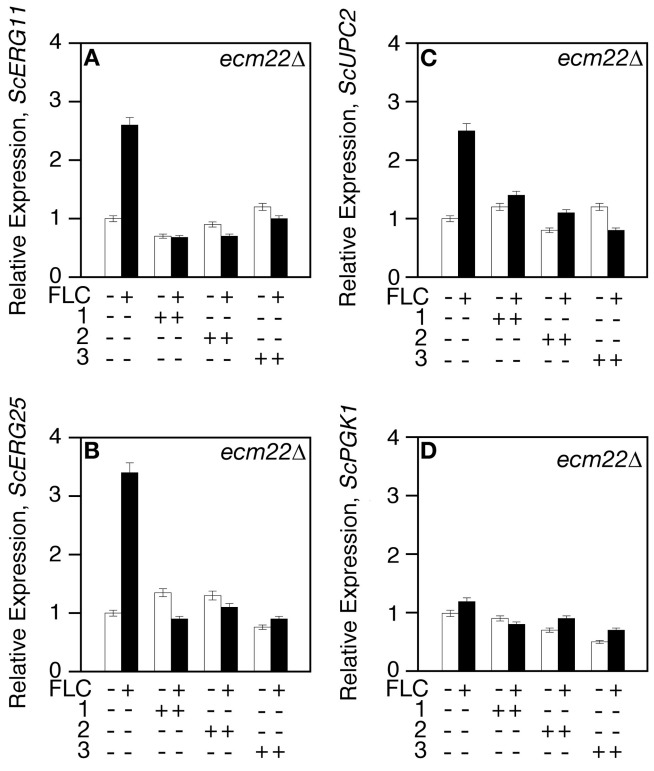

FIG 5 Compounds 1, 2, and 3 inhibit fluconazole-induced Upc2-dependent activity. Cells were grown to exponential phase in the absence (−) and presence (+) of fluconazole (FLC) and each compound (compound 1, 2, or 3) for 6 h at 30°C. Total RNA was extracted, and the mRNA expression levels of *ERG11*, *ERG25*, *UPC2*, and *PGK1* from *S. cerevisiae* (*Sc*) were determined by qRT-PCR.

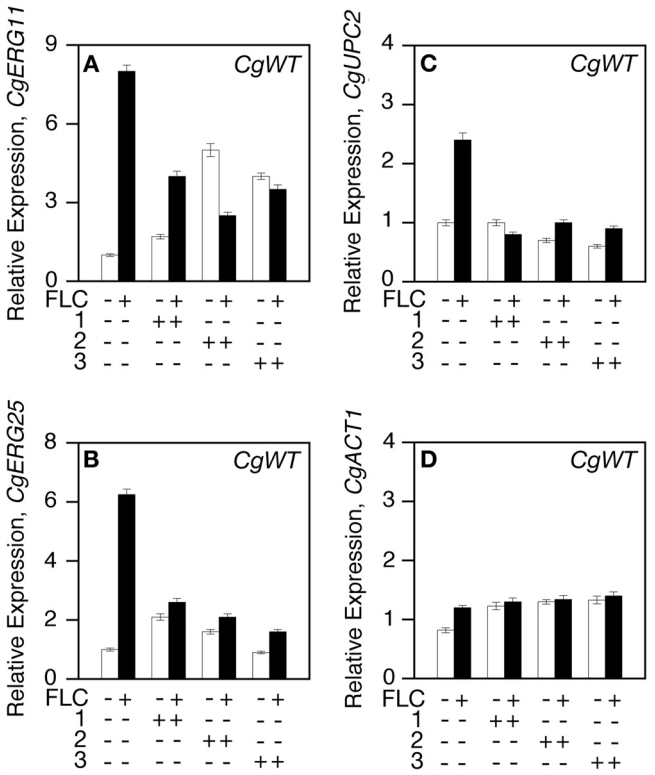

FIG 6 Compounds 1, 2, and 3 inhibit fluconazole-induced CgUpc2-dependent activity. Cells were grown to exponential phase in the absence (−) and presence (+) of fluconazole and each compound (compound 1, 2, or 3) for 6 h at 30°C. Total RNA was extracted, and the mRNA expression levels of *ERG11*, *ERG25*, *UPC2*, and *PGK1* from *C. glabrata* (*Cg*) were determined by qRT-PCR.

DISCUSSION

Many resistance mechanisms stimulate the overexpression of *ERG* genes, including gain-of-function mutations in *UPC2* (35, 37, 38, 56). The *UPC2* mutations responsible have been found in resistant clinical isolates, and resistance has been recapitulated in laboratory strains (37, 38). Upc2 is the most downstream factor regulating a pathway responsible for induction of *ERG* gene expression in the presence of azole antifungals. It represents an excellent target for compound discovery, as inhibition would circumvent multiple resistance mechanisms upregulating *ERG* genes (54).

A whole-cell HTS assay that was designed to identify compounds attenuating ScUpc2-dependent transcription was developed. An *in vitro* AlphaScreen assay was implemented to determine whether the compounds were true Upc2 DNA binding inhibitors. All compounds identified reduced Upc2 activity but did not inhibit DNA binding. Thus, the three compounds most likely attenuate a signaling pathway(s) regulating Upc2 function in response to a block in sterol biosynthesis. To our knowledge, these are the first small molecule Upc2 pathway inhibitors identified.

The compounds identified inhibited cell growth but did not kill cells. This seems reasonable since *Scecm22 Scupc2* double null cells are viable, although their growth is compromised (28, 29). Vegetative cell growth was inhibited in the absence of fluconazole, suggesting that Upc2 may regulate basal gene expression. A genome-wide ChIP study showed that CaUpc2 bound to promoters involved in ribosomal subunit biosynthesis, general transcription factor activities, and sulfur amino acid metabolism (31). The fact that wild-type *S. cerevisiae* cells were growth inhibited suggests the compounds regulate both ScUpc2 and ScEcm22. This is interesting, in light of the fact that ScUpc2 and ScEcm22 activities may not be completely redundant, as Ecm22 binding to SREs is reduced in the presence of fluconazole, while ScUpc2 binding is increased (47).

C. glabrata is more evolutionarily similar to *S. cerevisiae* than is *C. albicans* (57). *C. glabrata* contributes significantly to systemic infections in immunocompromised patients (58–60). Infections

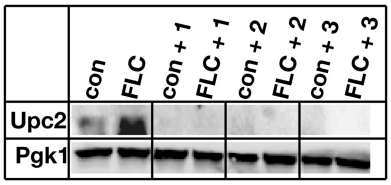

FIG 7 Compounds 1, 2, and 3 decrease Upc2 protein expression in the presence of fluconazole. Wild-type *S. cerevisiae* cells were grown to exponential phase in the absence and presence of fluconazole (FLC) and each compound (compound 1, 2, or 3) for 6 h at 30°C (con, control). Cell lysates were then prepared, and proteins were resolved by SDS-PAGE. The protein level of ScUpc2 was visualized by Western blot analysis using rabbit anti-ScUpc2 polyclonal antibodies and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG polyclonal antibodies.

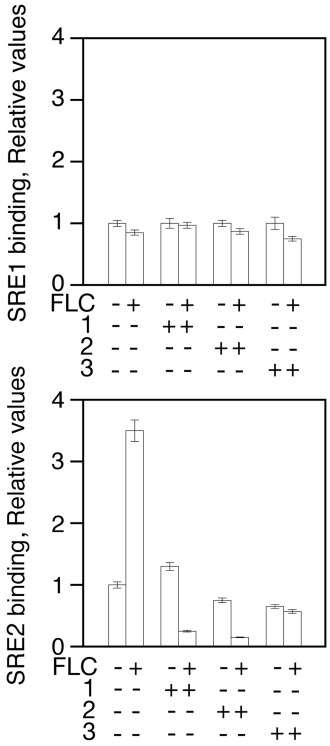

FIG 8 Compounds 1, 2, and 3 decrease Upc2 SRE binding in the presence of fluconazole. Wild-type *S. cerevisiae* cells were grown to exponential phase in the absence and presence of fluconazole and each compound for 6 h at 30°C. ScUpc2 was immunoprecipitated from cross-linked cells using rabbit anti-ScUpc2 polyclonal antibodies. Cross-linked ScUpc2-bound DNA was isolated and quantitated by qRT-PCR using primers specific for SRE1 or SRE2.

caused by *C. glabrata* are on the rise, due to acquisition of azole drug resistance (61, 62). *C. glabrata* cells did respond to drug treatment. We did not observe the same for *C. albicans*. CaUpc2 is highly orthologous to ScUpc2 and ScEcm22, with BLAST search values of e^{-112} and $2e^{-87}$, respectively (28). In addition, there is 66% and 64% homology within the N-terminal conserved Zn_2-Cys_6 binuclear cluster and C-terminal transmembrane domain of each protein, respectively (28). Resistant *C. albicans* strains have conserved *CaUPC2* gain-of-function mutations that are seen in *ScUPC2* alleles conferring resistance (35, 36, 38).

Based on this degree of homology and conservation of function, it is somewhat surprising that growth inhibition was not observed. Again, one possibility is that an *S. cerevisiae* strain carrying an *ScUPC2-LacZ* gene was used, rather than a *C. albicans* strain harboring a *CaUPC2-LacZ* gene. Compounds inhibiting *CaUpc2* alone could have possibly been missed. Another possibility is that *C. albicans* uses other transcription factors to regulate *ERG* gene expression in addition to CaUpc2 (32, 63). The CaTac1 transcription factor induces the expression of efflux pump genes needed for antifungal drug excretion (64–66). CaUpc2-dependent and -independent factors regulating *CaUPC2* expression also exist (32, 63). It is possible that targeting several pathways may be necessary to cause *C. albicans* growth inhibition. In any event, the compounds identified may serve as initial precursors for the chemical optimization of novel antifungal agents with widespread use in treating a number of serious systemic fungal infections.

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