

1      **Discovery of a novel class of orally active antifungal  $\beta$ -1,3-D-glucan synthase inhibitors**

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19      **Abstract:**

20      The echinocandins are a class of semisynthetic natural products that target  $\beta$ -1,3-glucan  
21      synthase (GS). Their proven clinical efficacy combined with minimal safety issues has made  
22      the echinocandins an important asset in the management of fungal infection in a variety of  
23      patient populations. However, the echinocandins are only delivered parenterally. A screen  
24      for antifungal bioactives combined with mechanism of action studies identified a class of  
25      piperazinyl-pyridazinones that target GS. The compounds exhibited in vitro activity  
26      comparable, and in some cases superior, to the echinocandins. The compounds inhibit GS in  
27      vitro and there was a strong correlation between enzyme inhibition and in vitro antifungal  
28      activity. In addition, like the echinocandins, the compounds caused leakage of cytoplasmic  
29      contents from yeast and produced a morphological response in moulds characteristic of GS  
30      inhibitors. Spontaneous mutants of *Saccharomyces cerevisiae* with reduced susceptibility to  
31      the piperazinyl-pyridazinones had substitutions in *FKS1*. The sites of these substitutions  
32      were distinct from those conferring resistance to echinocandins, likewise echinocandin-  
33      resistant isolates remained susceptible to the test compounds. Finally, we present efficacy  
34      and pharmacokinetic data on an example of the piperazinyl-pyridazinone compounds that  
35      demonstrated efficacy in a murine model of *Candida glabrata* infection.

36 **Introduction**

37 The echinocandins are the newest class of antifungal agents approved for the treatment of  
38 invasive fungal infections. There are now three echinocandins approved for clinical use,  
39 caspofungin (Cancidas®, Merck), micafungin (Mycamine®, Astellas), and anidulafungin  
40 (Eraxis®, Pfizer) and each is derived by semisynthetic modifications of naturally occurring  
41 lipopeptide antibiotics with molecular weights ranging from 1140-1292. The key features of  
42 the echinocandins that have made them a successful addition to antifungal treatment  
43 regimens are: 1) enhanced spectrum for *Candida* spp., including non-albicans *Candida*; 2)  
44 consistent fungicidal activity against *Candida* spp.; 3) improved hepatic and renal safety  
45 profile compared with the azoles and polyenes; and 4) reduced cytochrome-mediated drug-  
46 drug interactions compared with the azoles.

47 The molecular target of the echinocandins appears to be  $\beta$ -1,3-D-glucan synthase  
48 (GS), a membrane-associated protein complex required for the synthesis of  $\beta$ -1,3-D-glucan  
49 polymers that comprise the major component of the fungal cell wall. The drug target was  
50 identified from both biochemical and genetic studies. For example, cell-free GS assays were  
51 used to monitor the impact of inhibitors on incorporation of glucose from a radiolabeled  
52 precursor molecule, UDP-[<sup>14</sup>C]-D-glucose, into glucan polymers (8) and since the minimal  
53 GS complex has not been identified GS activity assays are performed using a crude  
54 membrane preparation. However, two subunits have been established as essential  
55 components of the GS complex; Fks1p and Rho1p in *Saccharomyces cerevisiae* (10, 28).  
56 Fks1p is a ~200 kDa integral membrane protein with as many as 16 membrane-spanning  
57 domains (9). Photoaffinity cross-linking studies with a substrate analog of UDP-glucose  
58 suggested that Fks1p is the catalytic subunit responsible for formation of the glycosidic

59 bonds (31). Rho1p, a Ras-like GTP-binding protein, is thought to be an essential regulator of  
60 GS activity (10, 28). Several studies have attempted to identify other members of the GS  
61 complex in yeast and other fungi, however the significance of these other proteins for  
62 enzyme function and regulation remains to be determined (4, 5, 13, 29, 31). Association and  
63 movement of Fks1p with actin patches also appears to be essential for proper cell wall  
64 integrity (35). With the dynamics of cell wall growth/remodelling and cell division  
65 intricately linked, many more candidate subunits or regulatory factors have been genetically  
66 associated with *FKS1* (18).

67 Genetic evidence that GS is the target of the echinocandins comes from the analysis  
68 of *S. cerevisiae* and *C. albicans* isolates that exhibit reduced susceptibility (25, 36). Two  
69 regions within Fks1p have been identified as hot spots for amino acid substitutions that cause  
70 high-level resistance to the echinocandins (24). These mutations confer a dominant  
71 resistance phenotype when expressed ectopically with a susceptible wildtype allele in *S.*  
72 *cerevisiae* or as a heterozygous allele in *C. albicans*. Clinical *Candida* spp. isolates with  
73 elevated MICs to the echinocandins also have mutations in the *FKS1* hot spots (25). In the  
74 moulds, the analysis has been more complex, as directed modification of *Fks1* in *A.*  
75 *fumigatus* can confer reduced susceptibility, although selection for resistance generally  
76 occurs in an as yet uncharacterized locus, not *Fks1* (12, 30).

77 The key limitation of the echinocandins is the requirement for administration by  
78 intravenous infusion, with little potential for development of oral formulations. Due to this  
79 dosing limitation, there remains significant interest in identifying new GS inhibitors  
80 unrelated to the echinocandins. One such class of inhibitor is the natural product, acid  
81 terpenoid enfumafungin which possesses in vitro activity similar to that of caspofungin (23).

82 Also, Kondoh et al. described a single, synthetic, piperazine propanol compound with  
83 antifungal activity that appears to target GS (16). While both of these GS inhibitors provide  
84 the potential for alternative formulations, to date neither has been demonstrated to have oral  
85 antifungal activity. Therefore, an orally bioavailable GS inhibitor with enhanced spectrum  
86 and fungicidal activity against *Candida* isolates would provide a valuable benefit for the  
87 treatment and prophylaxis of invasive fungal infection. An oral formulation would facilitate  
88 administration, particularly in an outpatient setting, and thus improve patient compliance and  
89 clinical outcome; it also offers the potential for combination therapy with an orally  
90 administered azole. Furthermore, a GS inhibitor that could be administered initially as an IV  
91 and then stepped down to an oral formulation would provide a clinical benefit over the  
92 echinocandins. In this paper we outline a drug discovery paradigm that was used to identify  
93 a novel class of fungal GS inhibitors and describe one compound with efficacy in a mouse  
94 model of *Candida* infection.

95 **Materials and Methods**

96 **Strains and growth media:** *Saccharomyces cerevisiae*, PM503 (*MAT $\alpha$  ade2 his3 $\Delta$ 200*  
97 *leu2 $\Delta$ 3,112 ura3-52  $\Delta$ pdr10::LEU2  $\Delta$ erg4::HIS3  $\Delta$ chs1, sal-hisG  $\Delta$ pdr5, sal-hisG*) was  
98 developed at Schering-Plough. JY101 (*MAT $\alpha$  ura3 leu2 his4*) contains the plasmid pLGSD5  
99 (+ATG)  $\text{GAL}_{\text{UAS}}::\text{lacZ}$  [*URA3*] (14, 27). *S. cerevisiae* strains S288C (*MAT $\alpha$  SUC2 gal2 mal*  
100 *mel flo1 flo8-1 hap1 ho bio1 bio6*) [ATCC Cat. No. 204508] and BY4742 (*MAT $\alpha$  his3 $\Delta$ 1*  
101 *leu2 $\Delta$ 0 lys2 $\Delta$ 0 ura3 $\Delta$ 0*) [ATCC Cat. No. 201389] were obtained from the American Type  
102 Culture Collection. *Candida albicans* BWP17 (*ura3/ura3 his1::hisG/his1::hisG*  
103 *arg4::hisG/arg4::hisG*) was provided by A. Mitchell (37). The *C. albicans* efflux mutant  
104 C697 (DSY1050; *Δcdr1::hisG/Δcdr1::hisG Δcdr2::hisG/Δcdr2::hisG Δmdr1::hisG-URA3-*  
105 *hisG/Δmdr1::hisG*) (3) and the wildtype progenitor, C693 (CAF2-1) were provided by D.  
106 Sanglard. All other strains were from the Schering-Plough Research Institute and Merck  
107 Research Laboratories strain collections. Media used were: YPD (per liter); 10g yeast  
108 extract, 20g Bacto peptone, 20g glucose, YPGalactose (20g/L of galactose instead of  
109 glucose), synthetic defined (SD) supplemented with appropriate amino acids and additives  
110 (MP Biomedicals, Solon, OH), RPMI (Invitrogen, Carlsbad, CA), and Sabaraoud dextrose  
111 agar (Difco, Becton, Dickinson, Franklin Lakes, NJ).

112 **Primary bioactive screen:** To prepare inoculum, PM503 was cultured from a glycerol stock  
113 onto a YPD + 80  $\mu$ g/mL uridine plate and incubated at 30°C until colonies appeared. A  
114 culture in YPD broth was started with a single colony of PM503 and grown overnight at  
115 30°C in a shaking incubator. The culture was then diluted to  $5 \times 10^3$  cells/mL in YPD and 45  
116  $\mu$ L of PM503 used to inoculate 96-well screening plates containing 5  $\mu$ L of compound, from  
117 the Schering-Plough compound collection, in 10% DMSO (1% DMSO final). Plates were

118 covered with a lid and incubated in a humidified incubator at 30°C for 48 hours and OD<sub>600nm</sub>  
119 was measured. Alternatively, following incubation 15 µL of colorimetric reagent (in dH<sub>2</sub>O:  
120 0.87 mg/mL 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-  
121 tetrazolium hydroxide (XTT), and 0.087 mg/mL phenazine methosulfate (PMS), Sigma  
122 Chemical Co.) was added to each well (15). In a total volume of 65 µL (compound,  
123 inoculum, and colorimetric reagent), the final concentrations are 200 µg/mL XTT and 20  
124 µg/mL PMS. The plates were incubated in the dark for 4-5 hr and then read at A<sub>492nm</sub> on a  
125 SpectraMax 340PC<sup>384</sup> Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA).  
126

127 **Microbroth susceptibility testing:** The yeast susceptibility testing procedure followed the  
128 CLSI document M27-A2 [*Reference Method for Broth Dilution Antifungal Susceptibility*  
129 *Testing of Yeasts; Approved Standard-Second Edition* (ISBN 1-56238-469-4); CLSI, 940  
130 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2002] with the  
131 following modifications: the final test volume was 100 µl and for testing *S. cerevisiae* strain  
132 PM503, YPD was used in place of RPMI 1640 broth. Fungicidal activity was measured  
133 following MIC determination. The contents of the first several clear wells were removed and  
134 plated on solid media lacking the test compound and plates were incubated at 30°C for 2  
135 days. The minimum fungicidal concentration (MFC) was judged to be lowest concentration  
136 of test compound that prevented regrowth. Filamentous fungi susceptibility testing  
137 procedure followed the CLSI document M38-A [*Reference Method for Broth Dilution*  
138 *Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard* (ISBN 1-56238-  
139 470-8). NCCLS, 940 West Valley Road, Suite 1400 Wayne, Pennsylvania 19087-1898 USA,  
140 2002] with the following modifications: the final test volume was 100 µl and the end point

141 used to assess the in vitro activity of GS inhibitors required a microscopic evaluation of cell  
142 morphology in the test wells (17). This endpoint, termed the minimum effective  
143 concentration (MEC), is characterized by changes in the fungal growth that resulted in  
144 truncated and highly branched hyphae.

145

146 **Permeabilized *S. cerevisiae* cells:** Permeabilization of yeast cells was performed according  
147 to Crotti, et al. (7), with some modifications. A 10 mL-starter culture of the *S. cerevisiae*  
148 strain PM503 in YPD medium with  $OD_{600}=3-4$  (1  $OD_{600} \sim 2-4 \times 10^7$  cells/mL) was used to  
149 inoculate 1 liter of YPD. The culture was grown at 30°C until  $OD_{600}=0.8$ . Cells were  
150 collected by centrifugation and resuspended in buffer (40 mM EDTA, 100 mM  $\beta$ -  
151 mercaptoethanol) at 1 g of cell pellet/3.5 mL buffer. The cell suspension was shaken for 30  
152 minutes at 30°C and the cells were centrifuged and washed with 5 mL 0.8 M sorbitol. Cells  
153 were resuspended in 6.8 mL of 2.9 mM citric acid, 11.3 mM dibasic sodium phosphate, 1  
154 mM EDTA, 0.8 M sorbitol, with constant shaking at 30°C for 30 minutes. After  
155 recentrifugation, the pellet was resuspended in 31.3 mL 50 mM Tris-HCl, pH 7.0, and  
156 incubated on ice for 5 minutes. Cells were again collected by centrifugation and then  
157 resuspended in 1 mL of 50 mM Tris-HCl and 33% glycerol, pH 7.5. The permeabilized cell  
158 preparation was stored at -80°C in aliquots.

159

160 **Cell membrane preparation:** Cell membranes were prepared as described (9) with minor  
161 modifications. One liter of YPD supplemented with 0.02 mg/mL adenine and 0.08 mg/mL  
162 uracil was inoculated with 10 mL of a saturated starter culture of *S. cerevisiae* PM503 or *C.*  
163 *albicans* strain BWP17 and grown at 30°C until  $OD_{600}$  reached 1. The cells were harvested

164 by centrifugation. After washing with 100 mL of breakage buffer (0.1 M potassium  
165 phosphate, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol, DTT), the cells were resuspended in  
166 50 ml ice-cold breakage buffer. The mixture was transferred to a Bead-Beater chamber  
167 (BioSpec Products, Bartlesville, OK) packed in ice. To each 50 mL sample was added 50 g  
168 of acid-washed glass beads (0.45  $\mu$ m, Sigma). The cells were disrupted using 12 x 20 second  
169 pulses with 2 minute-cooling intervals. Cell debris was removed by centrifugation at 3,000 g  
170 for 20 minutes at 4°C, and the supernatant was collected and centrifuged at 100,000 g for 1  
171 hour at 4°C to pellet the membrane fraction. The pellet was resuspended in 5 mL of ice-cold  
172 breakage buffer containing 25% glycerol, homogenized with a Dounce tissue homogenizer  
173 and the final preparation stored at –80°C in small aliquots.

174

175 **Glucan synthase assay:** GS activity was measured in 96 well Optiplates (PerkinElmer) as  
176 published (21, 33). To each well was added either 3  $\mu$ L of the test compound in 100%  
177 DMSO at 10x the final concentration, or 3  $\mu$ L of 30  $\mu$ g/mL caspofungin in 100% DMSO  
178 (positive control), or 3  $\mu$ L 100% DMSO (negative control). GS activity in either 2  $\mu$ L  
179 permeabilized PM503 cells or 3  $\mu$ L membrane preparation derived from either PM503 or  
180 BWP17 was then added to the plate. The reaction was initiated by adding 25  $\mu$ L reaction  
181 buffer (0.6 mM UDP-Glucose, 0.6 nCi [ $U$ - $^{14}$ C]UDP-Glucose [327 mCi/mmol, Amersham  
182 Bioscience], 20  $\mu$ M GTP- $\gamma$ -S, 25 mM Sodium Fluoride, 7.5 mg/mL BSA, 8% glycerol in 75  
183 mM Tris-HCl, pH 7.5). The assay plate was then incubated on a shaker for 1.5 hour at room  
184 temperature and the reactions were quenched by the addition of 250  $\mu$ L 1% trichloroacetic  
185 acid (TCA). The quenched reaction was mixed by pipetting, and immediately transferred to  
186 a 96-well filter plate (Glass fiber B on 0.65  $\mu$ m hydrophilic durapore membrane, Millipore)

187 pre-wetted with wash buffer (5% TCA, 60 mM sodium pyrophosphate). Newly synthesized,  
188 radio-labeled  $\beta$ -1,3-D-glucan polymers were collected on the filter membrane by applying a  
189 vacuum to the plate using a MutiScreen Resist Vacuum Manifold (Millipore). The filter  
190 plate was washed 4 times with 200  $\mu$ L wash buffer. The plate was then dried at 50°C for 30  
191 minutes. Microscint-0 (100  $\mu$ l, PerkinElmer) was added to each well and the plate was  
192 counted in a TopCount NXT plate reader (PerkinElmer).

193

194 **Cell wall integrity assay:** *S. cerevisiae* strain JY101 [pLGSD5 (+ATG) GAL<sub>UAS</sub>::lacZ] was  
195 used to inoculate YPD medium and incubated overnight at 30°C with shaking. A 50  $\mu$ L  
196 aliquot of the overnight culture was inoculated into 100 mL of YPGalactose and incubated at  
197 30°C with shaking for 24 hours. The cells were collected by centrifugation and resuspended  
198 in YPD to achieve an OD<sub>600</sub> of 0.6 and 50  $\mu$ L of this cell suspension was dispensed into each  
199 well of a filter plate (Millipore# MSHVN4B10) aligned with a catch plate beneath (Corning  
200 Costar #3370). The plate was covered and incubated in a humidified incubator at 30°C for  
201 3.5 to 4 hours. The test compounds (in 100% DMSO) were added to each well and the plate  
202 incubated for 1 hour at 30°C. The filter plate and attached catch plate were subjected to  
203 centrifugation at 2000 $\times$ g for 5 minutes to separate the cells from the media. To the wells  
204 containing filtrate 50  $\mu$ L of 2X Z Buffer (120 mM Na<sub>2</sub>HPO<sub>4</sub>, 80 mM NaH<sub>2</sub>PO<sub>4</sub>, 2 mM  
205 MgSO<sub>4</sub>, 20 mM KCl, pH=7.0, 1.6 mg/mL CPRG [chlorophenol red- $\beta$ -D-galactopyranoside  
206 monosodium salt, Roche Diagnostics #884308]) were added and the plate incubated at room  
207 temperature for 1 hour in the dark. Absorbance of each well was measured at 574 nm and  $\beta$ -  
208 galactosidase activity was calculated [(1000XA574) / (time<sub>min</sub>Xvol<sub>mL</sub>)].

209

210 **Resistant mutant selection:** *S. cerevisiae* strain S288C was grown to saturation in YPD  
211 broth and  $2 \times 10^7$  cells were spread on YPD agar plates containing SCH A (60  $\mu$ g/mL), plates  
212 were incubated at 30°C until colonies arose. Colonies were restreaked on YPD agar plates  
213 containing 60  $\mu$ g/mL SCH A. To determine the sequence of the *FKS1* gene in the resistant  
214 isolates, genomic DNA was prepared and amplified by PCR with primer sets producing  
215 overlapping products of approximately 2kb that spanned the entire gene. These PCR  
216 products were then sequenced. GS inhibitor-resistant alleles of *FKS1* were cloned into  
217 pRS426 by gap repair cloning (19). pRS426 was cleaved with KpnI/SacI, gel purified and  
218 used to co-transform W303 (ATCC Cat. No. 20060) along with two PCR fragments (A and  
219 B) spanning *FKS1* (overlapping by 500 bp) with homology to the 50 bp of the vector  
220 sequence immediately flanking the KpnI and SacI sites. Fragment A was produced with  
221 primers 5'-  
222 TCACGACGTTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTCGT  
223 GCTTGACTAAGACAAA-3' and 5'-CATTTCAGTTCATGTGGT-3'. Fragment B was  
224 produced with primers 5'-GGAAAGAATTCTGCTGTCAT-3' and 5'-  
225 GCTATGACCATGATTACGCCAAGCGCGCAATTAAACCTCACTAAAGGGAATACA  
226 TTCCTTCGGCAGATAG-3'. Plasmid DNA from transformants was prepared and used to  
227 transform *E. coli* for plasmid preparation and sequencing. A clone containing the correct  
228 *FKS1* sequence was then used to clone GS-resistant alleles. The R2 allele was amplified  
229 from chromosomal DNA with primers flanking the BglII and BspEI sites. The resulting  
230 fragment was cleaved with BglII/BstEI and cloned into BglII/BstEI cleaved pRS426-FKS1.  
231 The R8 allele was amplified from chromosomal DNA with primers flanking the BspEI and

232 NarI sites. The resulting fragment was cleaved with BspEI/NarI and ligated into BspEI/NarI  
233 cleaved pRS426-FKS1. The sequence of each insert was confirmed.

234

235 **In vivo models of fungal infection:** All animal studies were carried out in accordance with  
236 the institutional animal care and use committee in an Association for Assessment  
237 Accreditation of Laboratory Animal Care-accredited program. Mice, CF-1 (Charles River)  
238 males approximately 14-16 g, were immunocompromised by  $\gamma$ -irradiation (500 rads) 3 days  
239 prior to infection. *C. glabrata* strain C624 or stain C454 infections were done by tail vein  
240 injection of  $10^7$  CFU/mouse on day 0. Four hours postinfection (PI), animals were treated  
241 with SCH B formulated in 40% hydroxypropyl-beta-cyclodextrin (HPBCD) by oral gavage.  
242 CSP, prepared in 0.9% NaCl, was administered by intraperitoneal (ip) injection at 5 mg/kg.  
243 At 7 days PI, animals were euthanized by CO<sub>2</sub> asphyxiation and kidneys from each animal  
244 harvested. Kidneys were then homogenized in sterile saline and the homogenate was diluted  
245 and plated on Sabouraud dextrose agar. Colonies were counted after 2-3 days incubation at  
246 37°C. For pharmacokinetic studies, uninfected mice were given the indicated doses of SCH  
247 B and animals, two for each time point, were euthanized by CO<sub>2</sub> asphyxiation and blood was  
248 drawn by cardiac puncture. Blood components were separated by centrifugation and plasma  
249 samples were stored at -20°C prior to analysis. Compound quantitation in plasma samples  
250 was done by via a high-performance liquid chromatography/mass spectrometry procedure  
251 using SCH B as a reference standard (6).

252 **Results**

253 **Identification of a novel class of  $\beta$ -1,3 D glucan synthase inhibitors.** Bioactive molecules  
254 were initially identified in a high throughput screen of the Schering-Plough compound  
255 collection using *S. cerevisiae* PM503, a strain harboring mutations in the major drug efflux  
256 pumps and certain cell wall modifying enzymes. The hit rate was <0.1% and most of the hits  
257 were not active against wildtype strains of *S. cerevisiae*, *Candida* spp., or *Aspergillus*  
258 *fumigatus*. Bioactive compounds were tested against a panel of mechanism of action-focused  
259 screens, including an assay measuring incorporation of radiolabeled glucose into  $\beta$ -1,3 D-  
260 glucan. One compound class, the piperazine-substituted pyridazinones, specifically inhibited  
261 GS. SCH A (Figure 1) was the most potent member of the initial group having IC50s of 0.25  
262  $\mu$ g/mL and 4.6  $\mu$ g/mL against *S. cerevisiae* (PM503) and *C. albicans* (BWP17) GS,  
263 respectively. None of the compounds from this class inhibited chitin synthase (data not  
264 shown). SCH A had limited activity against wildtype *S. cerevisiae*, *Candida* spp. and *A.*  
265 *fumigatus* (Table 1); the exception was the clinical *C. glabrata* isolate, C624.

266 The activity of SCH A against the *A. fumigatus* clinical isolate, ND158, was  
267 measured as a minimum effective concentration (MEC); typical of the activity seen with the  
268 echinocandin class of GS inhibitors (17). Compound optimization efforts to improve the  
269 antifungal activity and pharmacological properties of this series produced several improved  
270 compounds including SCH B and SCH C (Fig. 1) (34, 38). In addition to antifungal activity,  
271 we observed that some compounds (e.g. SCH B) may be substrates for efflux. As shown in  
272 Table 1, the MIC of SCH B was lower for the efflux deficient C697 strain compared to the  
273 matched, efflux competent C693 strain. As a class, the piperazinyl-pyridazinones do not  
274 have antibacterial activity (data not shown).

275

276 **In vitro activity profile of the piperazine-substituted pyridazinones.** The antifungal  
277 activities of the piperazine-substituted pyridazinones were compared with caspofungin and  
278 fluconazole (an ergosterol biosynthesis inhibitor). Although the initial compound, SCH A,  
279 had limited activity and spectrum; chemical modification produced compounds, such as SCH  
280 C, with significantly improved activity and spectrum (Table 1). SCH B was fungicidal for *C.*  
281 *albicans* C43 and C693 (MFC = 25 µg/mL). Echinocandin-resistant *C. albicans* isolates with  
282 caspofungin MICs of >16 µg/mL remained susceptible to the piperazinyl-pyridazinones  
283 (Table 1). The mould activity of the piperazinyl-pyridazinones, measured as a MEC, was  
284 comparable to the echinocandins. However, unlike CSP, SCH C was active against  
285 *Cryptococcus neoformans*, *Fusarium moniliforme*, and *Trychophyton* spp.

286

287 **Mechanism of action and resistance of the piperazinyl-pyridazinone antifungal  
288 compounds.** Several lines of evidence indicate that the piperazinyl-pyridazinone compounds  
289 act through inhibition of GS. There was a significant ( $p<0.001$ ) correlation between activity  
290 in the biochemical assay (using membrane preparations) and antimicrobial activity; this  
291 correlation held true across a broad range of compound activities for both *S. cerevisiae* and  
292 *C. albicans* GS (Fig 2A). Exposure of susceptible filamentous fungi, such as *A. fumigatus*, to  
293 a GS inhibitor resulted in a distinct change in morphology (Figure 2B) that was characterized  
294 by substantially truncated and highly branched hyphae (17). First observed with the  
295 echinocandins and papulacandins, this morphological response is also seen upon exposure to  
296 another GS inhibitor, enfumafungin (23). The rigid fungal cell wall, composed mainly of  
297 glucan and chitin polymers, provides an essential barrier to osmotic stress. Inhibition of GS

298 compromises the integrity of the cell wall, ultimately resulting in cell lysis and leakage of  
299 cytoplasmic contents (2, 20). To monitor the impact of the GS inhibitors on cell wall  
300 integrity we used a strain of *S. cerevisiae* expressing  $\beta$ -galactosidase (Fig 2C). Exposure of  
301 this strain to 5-fluorocytosine, amphotericin B, aureobasidin, or posaconazole did not result  
302 in the release of  $\beta$ -galactosidase, in agreement with their mechanisms of action. Caspofungin  
303 and aculeacin, at concentrations near their MIC, lead to the release of  $\beta$ -galactosidase.  
304 Likewise, SCH A and an analog, SCH D, at concentrations near their MIC, also caused  
305 release of  $\beta$ -galactosidase.

306 Mutations conferring resistance to the echinocandins, in both laboratory generated  
307 mutants and in clinical isolates, have been localized to two regions of the *FKS1* gene; hot  
308 spot 1 and less commonly hot spot 2 (24). Using SCH A and the haploid *S. cerevisiae* strain  
309 S288C we selected for resistant isolates (approximately 1 per  $\sim 10^7$  cells plated). Two  
310 isolates (R2 and R8) were characterized further. Both isolates remained susceptible to  
311 caspofungin and fluconazole (Table 2). Sequencing the *FKS1* gene identified a mutation in  
312 each strain that resulted in single amino acid substitutions (R2; T1175S and R8; F1297L).  
313 To verify that these substitutions were responsible for the resistance phenotype the *FKS1*  
314 alleles were cloned into a shuttle vector, pRS426 (32) and introduced into a susceptible strain  
315 (BY4742/ $\Delta$ fks1). Cells containing the recombinant *FKS1* genes from the two resistant  
316 isolates, but not the wildtype allele, had higher MICs to the piperazinyl-pyridazinone GS  
317 inhibitors but not to caspofungin (Table 3). Similarly, inhibition of GS activity in  
318 membranes prepared from strain R2 required three-fold more piperazinyl-pyridazinones than  
319 the corresponding wildtype preparation; both membrane preparations were equally inhibited  
320 by caspofungin (Table 4). Also, the apparent Km of the R2 (Fks1[T1175S]p) enzyme for

321 UDP-glucose did not change substantially from the wildtype, while the Vmax of the wildtype  
322 enzyme was approximately 3-fold higher than the mutant enzyme.

323

324 **In vivo activity.** *C. glabrata* infection remains an important contributor to overall  
325 candidemia rates in the United States and Canada and with the increasing prevalence of azole  
326 resistance has become a recalcitrant pathogen (26). SCH B, which exhibited reasonable in  
327 vitro activity against *C. glabrata*, was tested in a model of systemic *C. glabrata* infection in  
328 immunocompromised mice (Figure 3). Each mouse was inoculated with  $10^7$  CFU of *C.*  
329 *glabrata* strain C624 (SCH B MIC = 0.1  $\mu$ g/mL) and dosed orally once a day with 50, 30, or  
330 10 mg/kg of SCH B. The 50 and 30 mg/kg doses were also split into either two or three  
331 doses/day. Following seven days of therapy *C. glabrata* levels in the kidneys were  
332 measured. The 50 mg/kg dose (either once a day or as a split dose) significantly reduced  
333 kidney burdens relative to the vehicle treated animals (Figure 3A). The 30 mg/kg dose,  
334 given as a single dose or split into two, also significantly reduced kidney burdens (Figure  
335 3A). Administering the 30 mg/kg in three 10 mg/kg doses diminished its activity compared  
336 to the other dosing regimens. Analysis of plasma levels in 10, 30, and 50 mg/kg (once daily)  
337 dosed animals revealed that the concentration of drug exceeded the MIC for at least 6-8  
338 hours (Figure 3B). In agreement with these findings, our estimation of the exposures  
339 achieved with the split doses showed that drug levels that were above the MIC for at least 8  
340 hours were the most efficacious. We next examined the activity of SCH B against a *C.*  
341 *glabrata* strain (C454) with a 10-fold higher MIC. CSP at 5 mg/kg, and the two high doses  
342 (100 and 50 mg/kg) of SCH B significantly reduced kidney fungal burdens relative to vehicle  
343 treated animals (Fig 3C). Measurement of blood levels of SCH B revealed that the 100, 50,

344 and 30 mg/kg doses achieved exposures (area under the curve) of 38, 11, and 6  $\mu\text{g} \cdot \text{hr}/\text{mL}$ ,  
345 respectively, and that the two efficacious doses resulted in plasma levels that exceeded the  
346 MIC of strain C454 for at least 4 hours (Figure 3D). The 30 mg/kg dose of SCH B did not  
347 achieve blood levels above the MIC and showed only a slight reduction in kidney burden.

348 **Discussion:**

349 A combination of classical screening for compounds with antifungal activity followed by  
350 directed mechanism of action studies identified a novel class of  $\beta$ -1,3 D glucan synthase  
351 inhibitors. Chemical modification of the original piperazinyl-pyridazinones leads identified  
352 several compounds with broad spectrum in vitro activity and efficacy in a murine model of  
353 *Candida* spp. infection. Like caspofungin, the compounds retained activity against yeast  
354 isolates that are resistant to fluconazole, but in contrast to caspofungin, at least one of the  
355 piperazinyl-pyridazinones displayed in vitro activity against *C. neoformans*, *F. moniliforme*,  
356 and *Trichophyton* spp. Activity against the moulds, like the echinocandins, was  
357 characterized by a morphological response, the "MEC" response, resulting in highly  
358 truncated and branched hyphae.

359 Several lines of evidence indicate that the antifungal activity of the piperazinyl-  
360 pyridazinones is a result of inhibition of GS. As mentioned above, like the echinocandins  
361 (and enfumafugin and the other terpenoids) the compounds induce the characteristic change  
362 in morphology in moulds such as *A. fumigatus*. Second, for both *S. cerevisiae* and *C.*  
363 *albicans* we observed a significant correlation among a collection of analogs of SCH A, B,  
364 and C between in vitro antimicrobial activity and inhibition of GS in a cell free assay. Third,  
365 both the piperazinyl-pyridazinones and caspofungin, at concentrations close to their MICs,  
366 caused leakage of recombinant, cytoplasmic  $\beta$ -galactosidase, suggesting that inhibition of GS  
367 significantly compromised cell wall integrity. Fourth, laboratory generated spontaneous  
368 mutants that were resistant to the piperazinyl-pyridazinones harbored mutations in *FKS1*, the  
369 gene encoding an essential subunit of GS. These mutations also resulted in an increased  
370 IC50 for the enzyme.

371        Mutations conferring resistance to the echinocandins are most commonly seen in two  
372    regions of the *FKS1* gene (25). These two hot spots flank what is predicted to be a large  
373    (~600 amino acids) cytoplasmic loop; the piperazinyl-pyridazinone resistance mutations  
374    described above mapped within this cytoplasmic loop. Consistent with the observation that  
375    the mutations clustered to separate domains of *FKS1*, there did not appear to be cross-  
376    resistance between the echinocandins and the piperazinyl-pyridazinones (Table 1). Based on  
377    a recent study of temperature sensitive *FKS1* mutants, a broad, highly conserved region of  
378    the cytoplasmic loop has been suggested to contain the catalytic domain (22). Mutations in  
379    this region producing a temperature sensitive growth phenotype resulted in GS activity with a  
380    reduced Vmax relative to the wildtype. The piperazinyl-pyridazinone resistance mutation R2  
381    (Fks1 [T1175S]) mapped to this region and when tested in vitro, the mutated protein  
382    exhibited a reduced Vmax relative to the wildtype (Table 4). However the kinetic data from  
383    the Okada et al. is from Fks1p derivatives containing multiple amino acid substitutions and a  
384    single amino acid substitution in hot spot 1 of *C. albicans* has also been shown to produce a  
385    significant (50%) reduction in the Vmax of the mutant GS relative to the wildtype (11).  
386    According to the topological model of Douglas et al. (9), echinocandin hot spot 1 resides  
387    within an inward facing loop adjacent to the large cytoplasmic domain that was not  
388    implicated in GS function by Okada et al. (22). Mutations in the less commonly encountered  
389    hot spot 2 are located within the broadly defined catalytic domain (22), but may be on an  
390    extracellular region separated from the piperazinyl-pyridazinones resistance mutations by a  
391    transmembrane domain.

392        A piperazinyl-pyridazinone GS inhibitor showed activity in murine model of fungal  
393    infection. *C. glabrata* is frequently encountered in the clinic, causing as many as 20% of all

394 candidemia cases in the United States and Canada (1). In a model of *C. glabrata* infection in  
395 immunocompromised mice, SCH B achieved a significant, dose-dependent reduction in  
396 kidney fungal burdens and this activity appeared to be correlated with the amount of time  
397 that the plasma concentration stayed above the MIC. With SCH B, efficacy against *C.*  
398 *glabrata* isolates with MICs of 0.1 and 1  $\mu$ g/mL was observed when plasma concentrations  
399 remained above the MIC for at least 4-8 hours. Further studies will be needed to identify the  
400 characteristics of the GS inhibitors that govern these initial pharmacokinetic and  
401 pharmacodynamic findings.

402 In summary we have identified a novel class of small molecule GS inhibitors with  
403 oral efficacy in a murine model of disseminated *C. glabrata* infection. The oral availability  
404 of these piperazinyl-pyridazinone GS inhibitors distinguishes them from the echinocandins;  
405 potentially allowing their use in step-down therapy from the hospital to the outpatient setting.  
406 In addition, with the candididal activity and efficacy of improved compounds, there may be  
407 opportunities to evaluate drug combinations with an azole having potent activity against  
408 moulds providing a highly useful broad spectrum tool for clinicians.

409

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415

416

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556  
557

## 558 TABLES

TABLE1. Antifungal properties of the novel piperazine-substituted pyridazinones

Organism	Strain #	MIC or MEC <sup>a</sup> (μg/mL)				
		SCH A	SCH B	SCH C	CSP <sup>b</sup>	FLZ <sup>c</sup>
<i>Candida albicans</i>	C43	>200	12.5	0.125	0.06	1
<i>C. albicans</i>	C375	>50	32	0.25	0.5	>64
<i>C. albicans</i>	C294			0.125	0.02	1
<i>C. albicans</i>	C693	100	6.25	0.06	0.06	0.5
<i>C. albicans</i>	C697		0.125	0.06	0.06	0.125
<i>C. albicans</i> (S645F) <sup>d</sup>	CLY16996		8	0.03	>16	0.25
<i>C. albicans</i> (S645P) <sup>d</sup>	CLY16997		8	0.03	>16	0.5
<i>C. dubliniensis</i>	C345	50		0.5	0.125	1
<i>C. glabrata</i>	C110		1	0.25	0.06	1
<i>C. glabrata</i>	C624	0.4	0.1	0.03	0.03	4
<i>C. glabrata</i>	C454	3.13	1	0.1	1	32
<i>C. parapsilosis</i>	C231	>200		0.25	0.125	4
<i>C. krusei</i>	C245	25		1	0.125	32
<i>Cryptococcus neoformans</i>	C237	>200		4	>16	16
<i>C. neoformans</i>	C297	>200		4	>16	16
<i>Saccharomyces cerevisiae</i>	C51			0.03	0.125	8
<i>Aspergillus flavus</i>	ND83	3.13		0.125	0.06	>64
<i>A. flavus</i>	ND255	3.13		0.03	0.06	>64
<i>A. fumigatus</i>	ND158	12.5	1.56	0.25	0.03	>64
<i>A. fumigatus</i>	ND231	6.25		0.5	0.125	>64
<i>A. fumigatus</i>	ND256	25		0.5	0.03	>64
<i>A. niger</i>	ND254	0.78		0.06	0.125	>64
<i>A. terreus</i>	ND125	25		0.5	0.06	>64
<i>Fusarium moniliforme</i>	ND244	3.13		0.03	>16	>64
<i>Rhizopus oryzae</i>	ND124	>200		>33	>16	>64
<i>Absidia corymbifera</i>	ND320	>200		>33	>16	>64
<i>Mucor circinelloides</i>	ND349	>200		>33	>16	>64
<i>Trichophyton mentagrophytes</i>	D30			0.06	>16	32
<i>T. rubrum</i>	D33	12.5		0.5	>16	64

559 <sup>a</sup>MIC100 (100% inhibition) shown for yeasts and MEC shown for moulds treated with GS  
 560 inhibitors. <sup>b</sup>Caspofungin (CSP). <sup>c</sup>Fluconazole (FLZ); reported as MIC50. <sup>d</sup>*CaFKS1* hotspot  
 561 1 mutations.

562

563

TABLE 2. In vitro susceptibility of piperazinyl-pyridazinone resistant *S. cerevisiae* isolates

Strain	MIC (µg/mL)		
	SCH A	CSP	FLZ
S288C (WT)	25	0.016	50
R2 ( <i>FKS1</i> [T1175S])	>100	0.016	50
R8 ( <i>FKS1</i> [F1297L])	>100	0.008	50

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565

TABLE 3. In vitro susceptibility of *S. cerevisiae* containing plasmid-bourne *FKS1* mutations

Strain	MIC (µg/mL)	
	SCH A	CSP
BY4742 ( <i>FKS1</i> )	12.5	0.03
BY4742 ( $\Delta$ <i>fks1</i> )	12.5	0.016
BY4742 ( $\Delta$ <i>fks1</i> )-pRS426[ <i>FKS1</i> ]	12.5	0.03
BY4742 ( $\Delta$ <i>fks1</i> )-pRS426[ <i>FKS1</i> (T1175S)]	100	0.03
BY4742 ( $\Delta$ <i>fks1</i> )-pRS426[ <i>FKS1</i> (F1297L)]	200	0.016

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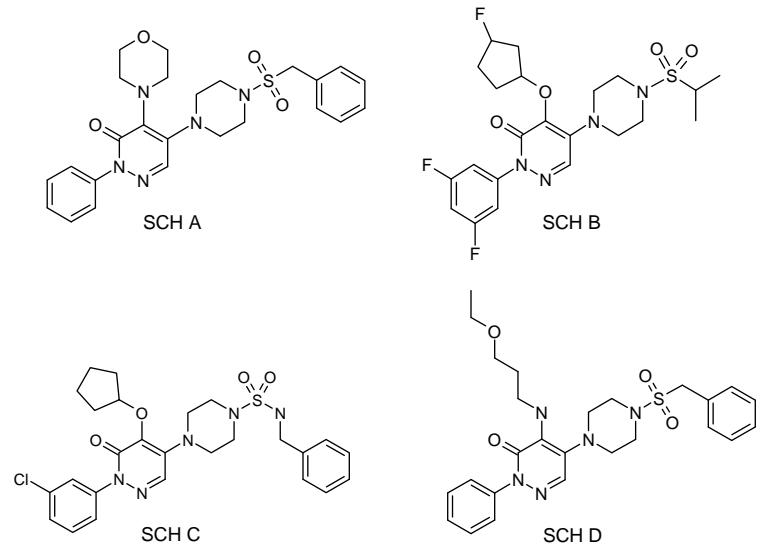
TABLE 4. GS properties of wildtype and piperazinyl-pyridazinone-resistant strains

Strain	IC50*	Km	Vmax
	(µg/mL)	(mM)	(nmol/min/mg)
S288C (WT)	0.22±0.08	0.72±0.11	28±0.9
R2 (Fks1[T1175S]p)	0.61±0.08	0.43±0.0.09	9.2±0.4

568 \*IC50 of SCH A

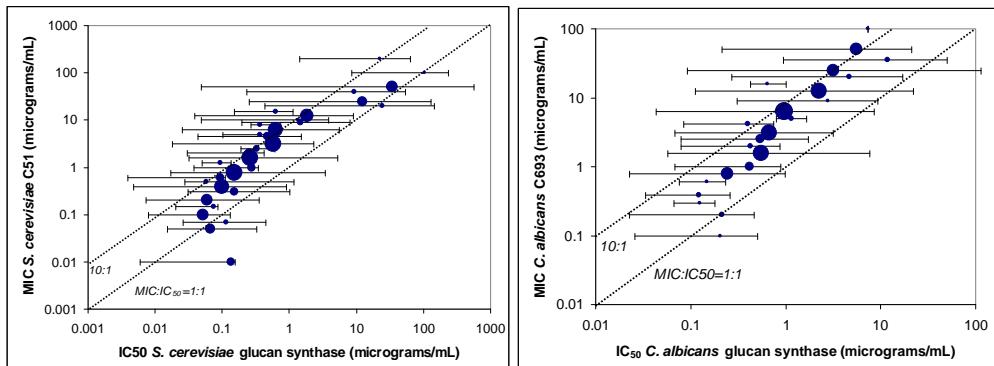
569 **FIGURES**

570



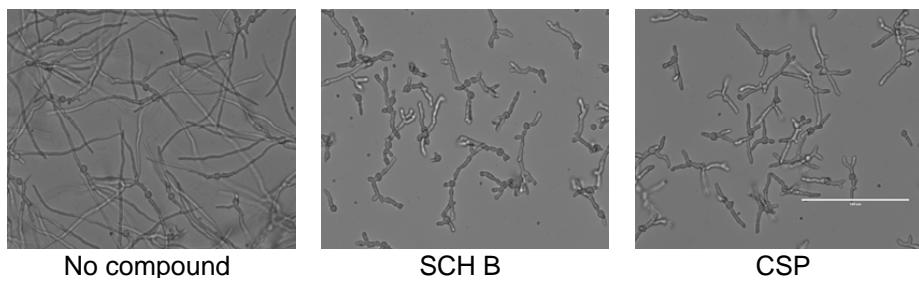
571 FIG. 1. Chemical structures of selected piperazine-substituted pyridazinone GS inhibitors.

572 A.



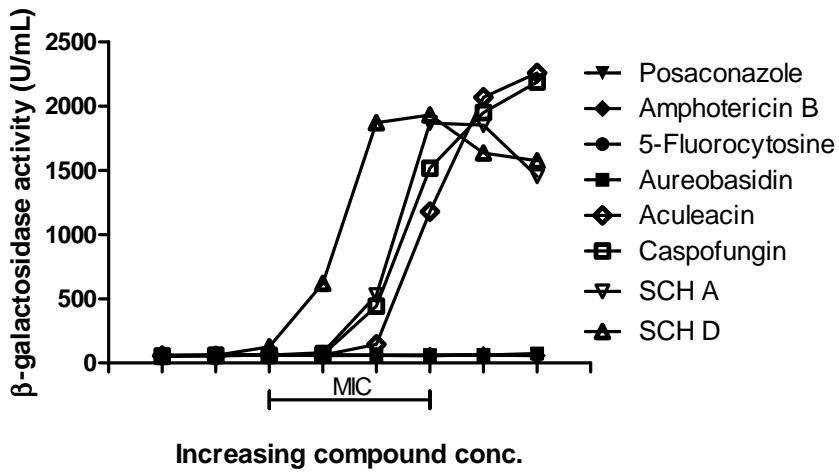
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574 B.



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576 C.



578 FIG. 2. Mechanism of action of the piperazine-substituted pyridazinones. (A) Correlation of  
579 *S. cerevisiae* MIC and GS IC50 (left panel) and *C. albicans* MIC and IC50 (right panel). For  
580 *S. cerevisiae* n=940 compounds, R=0.263 and p<0.001. For *C. albicans* n=534 compounds,  
581 R=0.268, and p<0.001. For both graphs, circle size depicts relative number of compounds in  
582 group. Dotted lines indicate 1:1 and 1:10 relationship between MIC and GS IC50  
583 Compounds produced as part of the pyridazinones optimization program (34, 38). (B)  
584 Morphology of *A. fumigatus* (ND158) treated with 1% DMSO, 6  $\mu$ g/mL SCH B, or 0.3  
585  $\mu$ g/mL CSP for 48 hrs. in RPMI. Scale bar = 100  $\mu$ m. (C) Leakage of cytoplasmic  $\beta$ -  
586 galactosidase from *S. cerevisiae* treated with the indicated compounds. Concentrations of  
587 each compound (2-fold dilution series) were adjusted to place the MIC of each compound  
588 within the range indicated.

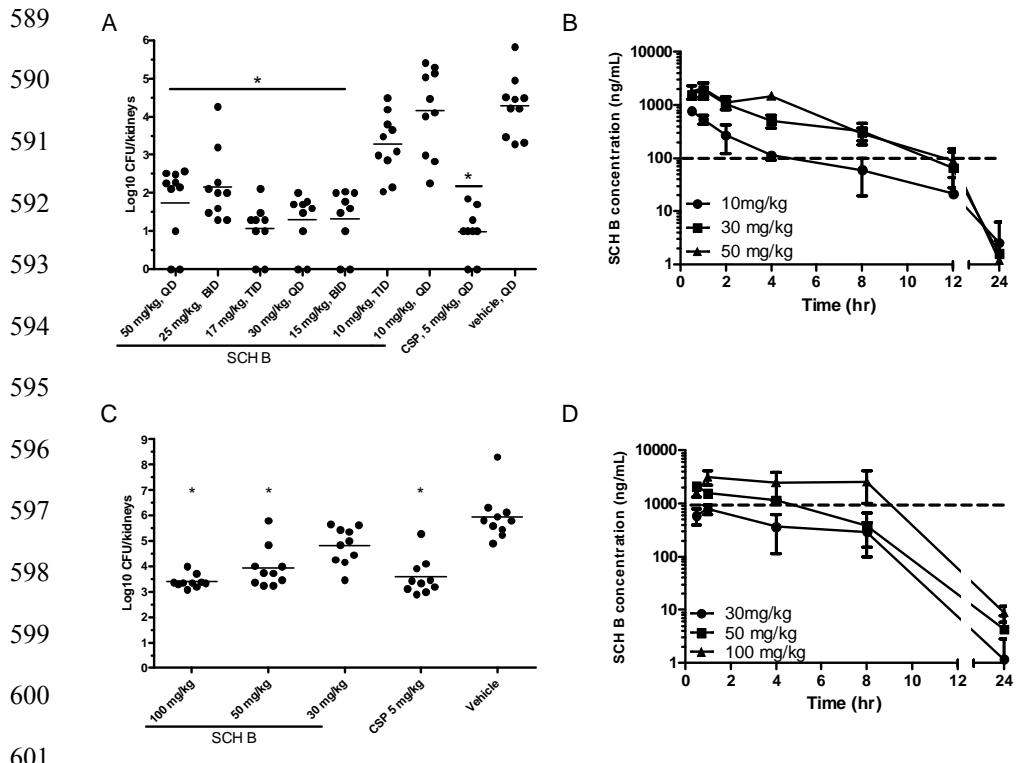


FIG. 3. Oral efficacy of a piperazinyl-pyridazinone GS inhibitor in a murine model of *C. glabrata* infection. (A) Kidney burdens of animals infected with *C. glabrata* C624. Compounds were dosed orally once per day (QD), twice per day (BID) or three times per day (TID). (B) SCH B plasma concentrations following the indicated single doses. Dashed line indicates C624 in vitro MIC (0.1  $\mu$ g/mL). (C) Kidney burdens of animals infected with *C. glabrata* C454. Compounds were dosed orally once per day at the indicated levels. (D) SCH B plasma concentrations following the indicated single doses. Dashed line indicates C454 in vitro MIC (1  $\mu$ g/mL). Asterisk indicates  $p < 0.001$ . Throughout these seven day infection/treatment studies no toxicity was observed for SCH B.