

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

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19 **Abstract:**

20 The echinocandins are a class of semisynthetic natural products that target β -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 *FKSI*. 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 β -1,3-D-glucan synthase
48 (GS), a membrane-associated protein complex required for the synthesis of β -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-[¹⁴C]-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 *FKSI* (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 *FKSI* hot spots (25). In the
74 moulds, the analysis has been more complex, as directed modification of *FksI* in *A.*
75 *fumigatus* can confer reduced susceptibility, although selection for resistance generally
76 occurs in an as yet uncharacterized locus, not *FksI* (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 indentifying 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 α* *ade2 his3- Δ 200*
97 *leu2-3,112 ura3-52 Δ pdr10::LEU2 Δ erg4::HIS3 Δ chs1, sal-hisG Δ pdr5, sal-hisG*) was
98 developed at Schering-Plough. JY101 (*MAT α* *ura3 leu2 his4*) contains the plasmid pLGSD5
99 (+ATG) GAL_{UAS}::lacZ [*URA3*] (14, 27). *S. cerevisiae* strains S288C (*MAT α* *SUC2 gal2 mal*
100 *mel flo1 flo8-1 hap1 ho bio1 bio6*) [ATCC Cat. No. 204508] and BY4742 (*MAT α* *his3 Δ 1*
101 *leu2 Δ 0 lys2 Δ 0 ura3 Δ 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; *Δ cdr1::hisG/ Δ cdr1::hisG Δ cdr2::hisG/ Δ cdr2::hisG Δ mdr1::hisG-URA3-*
105 *hisG/ Δ mdr1::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 μ 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 5×10^3 cells/mL in YPD and 45
116 μ L of PM503 used to inoculate 96-well screening plates containing 5 μ 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_{600nm}
119 was measured. Alternatively, following incubation 15 µL of colorimetric reagent (in dH₂O:
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_{492nm} on a
125 SpectraMax 340PC³⁸⁴ 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 β -
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 μ 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 μ L of the test compound in 100%
177 DMSO at 10x the final concentration, or 3 μ L of 30 μ g/mL caspofungin in 100% DMSO
178 (positive control), or 3 μ L 100% DMSO (negative control). GS activity in either 2 μ L
179 permeabilized PM503 cells or 3 μ L membrane preparation derived from either PM503 or
180 BWP17 was then added to the plate. The reaction was initiated by adding 25 μ L reaction
181 buffer (0.6 mM UDP-Glucose, 0.6 nCi [U-¹⁴C]UDP-Glucose [327 mCi/mmol, Amersham
182 Bioscience], 20 μ M GTP- γ -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 μ 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 μ m hydrophilic durapore membrane, Millipore)

187 pre-wetted with wash buffer (5% TCA, 60 mM sodium pyrophosphate). Newly synthesized,
188 radio-labeled β -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 μ L wash buffer. The plate was then dried at 50°C for 30
191 minutes. Microscint-0 (100 μ 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_{UAS}::lacZ] was
195 used to inoculate YPD medium and incubated overnight at 30°C with shaking. A 50 μ 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 OD₆₀₀ of 0.6 and 50 μ 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 μ L of 2X Z Buffer (120 mM Na₂HPO₄, 80 mM NaH₂PO₄, 2 mM
205 MgSO₄, 20 mM KCl, pH=7.0, 1.6 mg/mL CPRG [chlorophenol red- β -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 β -
208 galactosidase activity was calculated [(1000XA574) / (time_{min}Xvol_{mL})].

209

210 **Resistant mutant selection:** *S. cerevisiae* strain S288C was grown to saturation in YPD
211 broth and 2×10^7 cells were spread on YPD agar plates containing SCH A (60 $\mu\text{g}/\text{mL}$), plates
212 were incubated at 30°C until colonies arose. Colonies were restreaked on YPD agar plates
213 containing 60 $\mu\text{g}/\text{mL}$ 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 TCACGACGTTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTTCGT
223 GCTTGACTAAGACAAA-3' and 5'-CATTTTCCAGTTCATGTGGT-3'. Fragment B was
224 produced with primers 5'-GGAAAGAATTCTGCTGTCAT-3' and 5'-
225 GCTATGACCATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAATACA
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 γ -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 CO₂ 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 CO₂ 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 β -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 β -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 μ g/mL and 4.6 μ 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 *Trichophyton* 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 β -galactosidase (Fig 2C). Exposure of
301 this strain to 5-fluorocytosine, amphotericin B, aureobasidin, or posaconazole did not result
302 in the release of β -galactosidase, in agreement with their mechanisms of action. Caspofungin
303 and aculeacin, at concentrations near their MIC, lead to the release of β -galactosidase.
304 Likewise, SCH A and an analog, SCH D, at concentrations near their MIC, also caused
305 release of β -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 *FKSI* 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 *FKSI* 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 *FKSI*
314 alleles were cloned into a shuttle vector, pRS426 (32) and introduced into a susceptible strain
315 (BY4742/ Δ fk1). Cells containing the recombinant *FKSI* 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 K_m of the R2 (Fks1[T1175S]p) enzyme for

321 UDP-glucose did not change substantially from the wildtype, while the V_{max} 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 $\mu\text{g/mL}$) 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 β -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 β -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 *FKSI*, 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 *FKSI* 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 *FKSI*, 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 *FKSI* 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 µg/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 candidicidal 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

TABLE1. Antifungal properties of the novel piperazine-substituted pyridazinones

Organism	Strain #	MIC or MEC ^a (µg/mL)				
		SCH A	SCH B	SCH C	CSP ^b	FLZ ^c
<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) ^d	CLY16996		8	0.03	>16	0.25
<i>C. albicans</i> (S645P) ^d	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 ^aMIC100 (100% inhibition) shown for yeasts and MEC shown for moulds treated with GS
 560 inhibitors. ^bCaspofungin (CSP). ^cFluconazole (FLZ); reported as MIC50. ^d*CaFKSI* hotspot
 561 1 mutations.
 562

563

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

Strain	MIC ($\mu\text{g/mL}$)		
	SCH A	CSP	FLZ
S288C (WT)	25	0.016	50
R2 (<i>FKSI</i> [T1175S])	>100	0.016	50
R8 (<i>FKSI</i> [F1297L])	>100	0.008	50

564

565

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

Strain	MIC ($\mu\text{g/mL}$)	
	SCH A	CSP
BY4742 (<i>FKSI</i>)	12.5	0.03
BY4742 ($\Delta fks1$)	12.5	0.016
BY4742 ($\Delta fks1$)-pRS426[<i>FKSI</i>]	12.5	0.03
BY4742 ($\Delta fks1$)-pRS426[<i>FKSI</i> (T1175S)]	100	0.03
BY4742 ($\Delta fks1$)-pRS426[<i>FKSI</i> (F1297L)]	200	0.016

566

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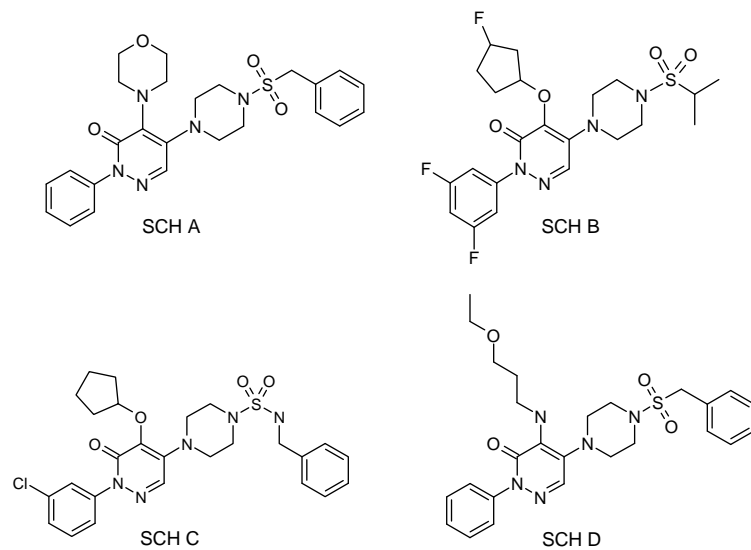
TABLE 4. GS properties of wildtype and piperazinyl-pyridazinone-resistant strains

Strain	IC50* ($\mu\text{g/mL}$)	Km (mM)	Vmax (nmol/min/mg)
S288C (WT)	0.22 \pm 0.08	0.72 \pm 0.11	28 \pm 0.9
R2 (Fks1[T1175S]p)	0.61 \pm 0.08	0.43 \pm 0.09	9.2 \pm 0.4

568

*IC50 of SCH A

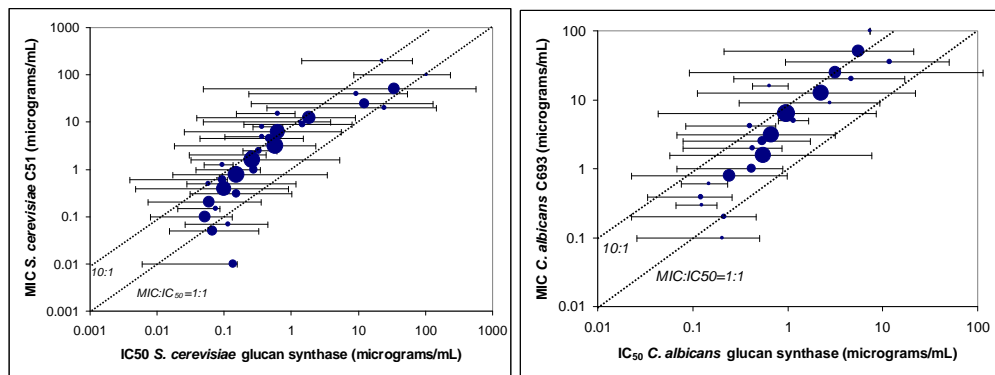
569 FIGURES



570

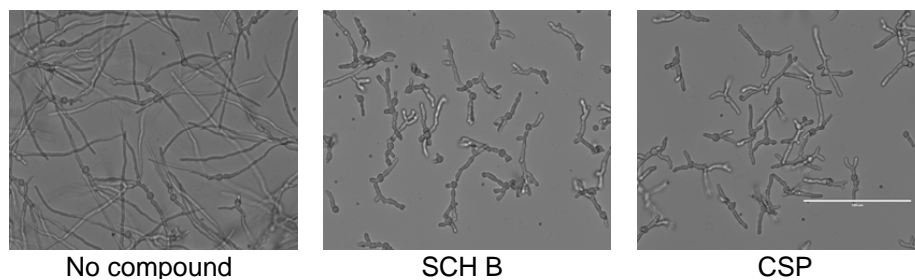
571 FIG. 1. Chemical structures of selected piperazine-substituted pyridazinone GS inhibitors.

572 A.



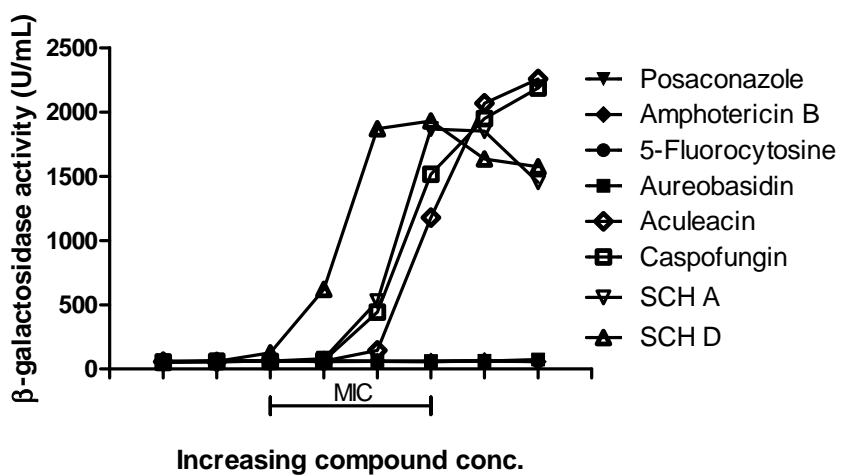
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574 B.



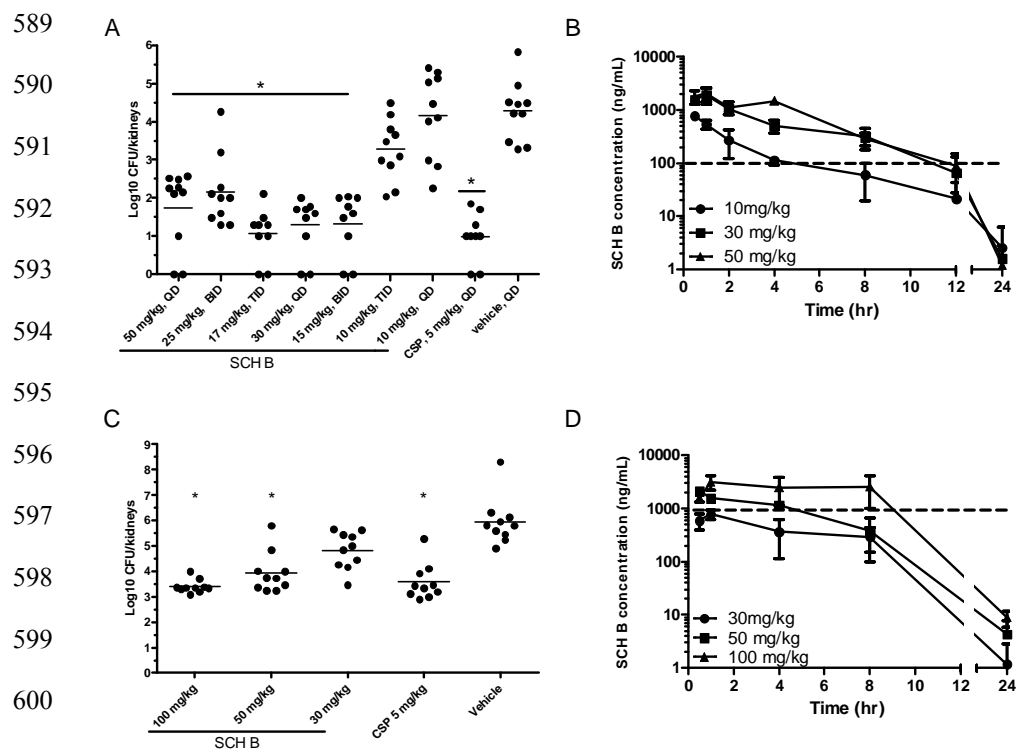
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576 C.



577

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 µg/mL SCH B, or 0.3
585 µg/mL CSP for 48 hrs. in RPMI. Scale bar = 100 µm. (C) Leakage of cytoplasmic β-
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.



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