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### Paper:

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AAC (Research Article, T.Colley)

1 Title

2 ***In vitro* and *in vivo* antifungal profile of a novel and long acting inhaled azole,**  
3 **PC945, on *Aspergillus fumigatus* infection**

4

5

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21 Running title: Antifungal activity of novel azole PC945

22 Key words: *Aspergillus fumigatus*, azole, inhalation, CYP51, azole resistant

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24 **ABSTRACT**

25

26 The profile of PC945, a novel triazole antifungal, designed for administration via inhalation, has  
27 been assessed in a range of *in vitro* and *in vivo* studies. PC945 was characterized as a potent,  
28 tight-binding inhibitor of *Aspergillus fumigatus* sterol 14 $\alpha$ -demethylase (CYP51A and CYP51B)  
29 activity (IC<sub>50</sub>, 0.23  $\mu$ M and 0.22  $\mu$ M, respectively), with characteristic type II azole binding  
30 spectra. Against 96 clinically isolated *A. fumigatus* strains, the MIC values of PC945 ranged  
31 from 0.032~>8  $\mu$ g/ml, whilst those of voriconazole ranged from 0.064~4  $\mu$ g/ml.  
32 Spectrophotometric analysis of the effects of PC945 against itraconazole-susceptible and -  
33 resistant *A. fumigatus* growth, yielded IC<sub>50</sub> (OD) values between 0.0012~0.034  $\mu$ g/ml, whereas  
34 voriconazole (0.019~>1  $\mu$ g/ml) was less effective than PC945. PC945 was effective against a  
35 broad spectrum of pathogenic fungi (MIC ranged from 0.0078~2  $\mu$ g/ml) including *Aspergillus*  
36 *terreus*, *Trichophyton rubrum*, *Candida albicans*, *Candida glabrata*, *Candida krusei*,  
37 *Cryptococcus gattii*, *Cryptococcus neoformans* and *Rhizopus oryzae* (1~2 isolates each). In  
38 addition, when *A. fumigatus* hyphae or human bronchial cells were treated with PC945, and then  
39 washed, PC945 was found to be quickly absorbed into both target and non-target cells and to  
40 produce persistent antifungal effects. In temporarily neutropenic immunocompromised mice  
41 infected with *A. fumigatus* intranasally, 50% of the animals survived until day 7 when treated  
42 intranasally with PC945 at 0.56  $\mu$ g/mouse, while posaconazole showed similar effects (44%) at  
43 14  $\mu$ g/mouse. This profile affirms that topical treatment with PC945 should provide potent  
44 antifungal activity in the lung.

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47 **INTRODUCTION**

48

49 The current management of the three major forms of aspergillosis: invasive aspergillosis  
50 (IA), chronic pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis  
51 (ABPA) (1-4), involves prophylactic or therapeutic administration of triazoles and, infrequently,  
52 surgical intervention (5). Existing antifungal medicines are predominantly dosed either orally or  
53 systemically. These frequently exploited routes of delivery are poor for treating airway disease,  
54 since drug concentrations achieved at the site of infection tend to be lower than those in other,  
55 healthy organs. This is especially so for the liver, which is a site of triazole toxicity: up to 15% of  
56 patients treated with voriconazole experience raised transaminase levels (6, 7). Exposure of the  
57 liver also results in significant drug interactions arising from triazole inhibition of hepatic P450  
58 enzymes (8, 9).

59 It is evident that there is an unmet clinical need for improved antifungal therapies which elicit  
60 fewer drug interactions; show reduced toxicity; achieve higher and more sustained pulmonary  
61 drug concentrations and also demonstrate potent activity against azole-resistant *Aspergillus*  
62 strains. Thus, there are several advantages of topical treatment over oral/systemic treatment  
63 which alter the risk benefit ratio of treatment favourably. An optimised compound for topical  
64 delivery should have prolonged lung tissue residence with limited systemic exposure to display a  
65 better adverse effect profile and eradicate invasive aspergillosis due to high concentration  
66 exposure. We have undertaken an extensive lead optimization program in order to identify potent  
67 azole antifungal agents with optimal properties for topical administration to the lung including  
68 tissue retention and physicochemical properties required for formulation. In this report we  
69 disclose the *in vitro* and *in vivo* activity of PC945, which is: 4-[4-(4-{{(3R,5R)-5-(2,4-

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70 difluorophenyl)-5-(1*H*-1,2,4-triazol-1-ylmethyl)oxolan-3-yl]methoxy}-3-  
71 methylphenyl)piperazin-1-yl]-*N*-(4-fluorophenyl)benzamide (Structure in Fig. 1A); a novel,  
72 triazole, antifungal agent designed specifically for inhaled administration (10).  
73

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## 74 MATERIALS AND METHODS

75 **Antifungal agents.** For *in vitro* antifungal assays, stock solutions of test agents were prepared in  
76 DMSO (2000 µg/ml). For *in vivo* studies test agents were suspended in physiological saline.  
77 PC945 was synthesised by Sygnature Discovery Ltd (Nottingham, UK), and voriconazole  
78 (Tokyo Chemical Industry UK Ltd., Oxford, UK), posaconazole (Apichem Chemical  
79 Technology Co., Ltd., Zhejiang, China), itraconazole (Arkopharma, Carros, France),  
80 amphotericin B (Selleckchem, Munich, Germany) and caspofungin (Selleckchem, Munich,  
81 Germany) were procured from commercial sources.

82

83 ***A. fumigatus* CYP51 binding assay and enzyme inhibitory activity.** *A. fumigatus* CYP51  
84 binding properties were determined as described by Warrilow *et al.* (11). Test agents were  
85 titrated against 4 µM recombinant *A. fumigatus* CYP51A or CYP51B proteins and binding  
86 saturation curves were constructed from the change in the absorbance between the spectral peak  
87 and the trough. A rearrangement of the Morrison equation was used to determine the dissociation  
88 constant ( $K_d$ ) values when ligand binding was tight (12).

89 A CYP51 reconstitution assay system was used to determine 50% inhibitory ( $IC_{50}$ )  
90 concentrations (13). Test agent was added to a mixture of 0.5 µM CYP51, 1 µM *A. fumigatus*  
91 cytochrome p450 reductase isoenzyme 1 (AfCPR1), 50 µM eburicol, 4% ( $w/v$ ) 2-hydroxypropyl-  
92 β-cyclodextrin, 0.4 mg ml<sup>-1</sup> isocitrate dehydrogenase, 25 mM trisodium isocitrate, 50 mM NaCl,  
93 5 mM MgCl<sub>2</sub> and 40 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) (pH ~7.2). The  
94 mixtures were then incubated at 37°C for 10 minutes prior to initiation with 4 mM β-NADPHNa<sub>4</sub>  
95 followed by shaking for 20 minutes at 37°C. Sterol metabolites were recovered by extraction  
96 with ethyl acetate followed by derivatisation with 0.1 ml *N,O*-

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97 bis(trimethylsilyl)trifluoroacetamide : trimethylchlorosilane (99:1) and 0.3 ml anhydrous  
98 pyridine prior to analysis by gas chromatography mass spectrometry.

99

100 **A. fumigatus sterol analysis.** A working suspension of *A. fumigatus* spores was prepared in  
101 filter-sterilised MOPS RPMI-1640 (RPMI-1640 containing 2 mM L-glutamine, 2% glucose,  
102 0.165 M MOPS, buffered to pH 7 with NaOH) at a final concentration of  $8 \times 10^6$  spores ml<sup>-1</sup>. To  
103 each 100 mm Petri dish, 10 ml of the working suspension was added and the dishes were  
104 incubated for 4 h at 35°C and 5% CO<sub>2</sub>. Samples for baseline determinations were collected by  
105 scraping, pelleted by centrifugation at 2000 rpm for 5 minutes and stored at -80°C. Test  
106 compounds or DMSO (50 µL) were added to the remaining dishes, which were subsequently  
107 gently rocked by hand to disperse the compounds. Dishes were incubated for 2 h at 35°C and 5%  
108 CO<sub>2</sub>. Samples were collected and processed as described above. Posaconazole and PC945  
109 concentrations of 0.0001, 0.001, 0.01, 0.1 and 1 µg ml<sup>-1</sup> were tested. These samples were  
110 prepared in the laboratory at Pulmocide Ltd., and sent to the laboratory in the Centre for  
111 Cytochrome P450 Biodiversity, Institute of Life Science, Swansea University Medical School,  
112 for experimentation.

113 Non-saponifiable lipids were extracted as previously reported (14) and were derivatised  
114 with 0.1 ml *N,O*-bis(trimethylsilyl)trifluoroacetamide : trimethylchlorosilane (99:1) and 0.3 ml  
115 anhydrous pyridine (2 h at 80°C) prior to analysis by gas chromatography mass spectrometry  
116 (15). Sterol composition was calculated using peak areas from the gas chromatograms and the  
117 mass fragmentation pattern compared to known standards were used to confirm sterol identity.  
118 The sterol content of *A. fumigatus* (basal) and treated *A. fumigatus* (either DMSO, posaconazole  
119 or PC945) were determined.

120

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121 **A. fumigatus cell based ergosterol assay.** Growth medium (RPMI-1640, 2 mM L-glutamine,  
122 2% glucose, 0.165 M MOPS, 0.5% BSA, pH 7.0) was added across a 96-well plate and test  
123 agents were added in duplicate. *A. fumigatus* (NCPF2010) conidia were added across the plate at  
124 a final concentration of  $1 \times 10^4 \text{ ml}^{-1}$ . After incubation for 24 h at 35°C, media was removed from  
125 all wells and replaced with reaction buffer (Amplex red cholesterol assay kit, ThermoFisher,  
126 A12216) and Amplex red solution. Plates were incubated for 30 minutes at 37°C, protected from  
127 light, after which fluorescence was quantified using a spectrophotometer. Media was removed  
128 from all wells and replaced with crystal violet solution (1% v/v), and plates were incubated at  
129 room temperature on a shaker for 30 minutes. Plates were washed three times with PBS, and  
130 sodium dodecyl sulfate solution (0.1% v/v) was added across the plate to lyse the cells. After  
131 incubation at room temperature for 1 h, absorbance was measured at OD<sub>590</sub> using a  
132 spectrophotometer.

133

134 **Fluorescent imaging of *A. fumigatus* infected cells.** Human alveolar epithelial cells (A549)  
135 were seeded onto collagen-coated coverslips and incubated at 37°C and 5% CO<sub>2</sub> for 24 h. Cells  
136 were incubated in the presence of test agents for 2 h, after which the media was replaced and the  
137 coverslips were incubated at 37°C, and 5% CO<sub>2</sub> for 24 h. CellTracker red CMTPX dye  
138 (ThermoFisher, C34552) was added to cell media for 30 minutes, wells were washed with PBS,  
139 and GFP-*A. fumigatus* conidia, [a kind gift from Professor William Hope, University of  
140 Liverpool], were added to wells at a final concentration of  $1 \times 10^3 \text{ spores ml}^{-1}$ . After 24 h  
141 incubation at 35°C and 5% CO<sub>2</sub>, coverslips were washed and affixed to slides using  
142 Fluoroshield™ with DAPI (Sigma, F6057).

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144 ***In vitro* antifungal activity against *A. fumigatus*.** Assessment of antifungal activity against a  
145 selection of *A. fumigatus* laboratory/clinical strains (NCPF2010 [National Collection of  
146 Pathogenic Fungi (NCPF), Bristol, UK], AF72 [NCPF, Bristol, UK], AF91 [NCPF, Bristol, UK],  
147 AF293 [NCPF, Bristol, UK], AF294 [NCPF, Bristol, UK], TR34-L98H [Professor Bretagne, St  
148 Louis Hospital, Paris, France]) was performed according to the European Committee on  
149 Antimicrobial Susceptibility Testing (EUCAST) definitive document EDef 9.3 (16), with the  
150 following exceptions: (i) 0.5% bovine serum albumin (BSA) was added to the growth medium to  
151 avoid any loss of lipophilic compounds by adherence to plastic plate surfaces and (ii) 384-well  
152 plates were used rather than 96-well plates. Growth medium (RPMI-1640, 2mM L-glutamine,  
153 2% glucose, 0.165 M MOPS, 0.5% BSA, pH 7.0) was added across the plate, test agents were  
154 added in quadruplicate and the DMSO concentration was identical across the plates. Conidia  
155 were added across the plate at a final concentration of  $1 \times 10^5 \text{ ml}^{-1}$ . Plates were incubated for 48  
156 h at 35°C after which turbidity was assessed by measuring optical density (OD) at 530 nm using  
157 a spectrophotometer, and the IC<sub>50</sub> and IC<sub>90</sub> values were calculated from the concentration-  
158 response curve generated for each test compound using a four-parameter logistic equation  
159 (Dotmatics, Bishops Stortford, UK). *A. fumigatus* ATCC204305 was used as the assay control.  
160 Determination of antifungal activity against 50 *A. fumigatus* clinical isolates from St Louis  
161 Hospital (Paris, France) was performed with 96-well plates using the modified EUCAST method  
162 in the presence of 0.5% BSA as described above. Antifungal susceptibility testing for 46 *A.*  
163 *fumigatus* isolates [obtained from the North West England Mycology Reference Centre] was  
164 performed by Evotec (UK) Ltd (Manchester, UK) according to EUCAST guidelines. Assessment  
165 of the antifungal activity of four of the *A. fumigatus* strains (ATCC1028, ATCC10894,  
166 ATCC13073, and ATCC16424) was performed according to methodology described by the

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167 Clinical and Laboratory Standards Institute (CLSI) by Eurofins Panlabs Taiwan Ltd. (Taipei,  
168 Taiwan).

169

170 ***In vitro* antifungal activity against other fungal species.** For the measurement of activity  
171 against *Cryptococcus gattii*, the method described in EUCAST definitive document EDef 7.2  
172 was used and assay plates were incubated statically at 37°C in ambient air for 24 h ( $\pm$  2 h) unless  
173 poor growth necessitated further incubation to 36 or 48 h (17). Antifungal potency against  
174 *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus terreus*, was determined as set out in  
175 EUCAST definitive document EDef 9.2 and assay plates were incubated at 37°C for 48 h (18).  
176 These tests were conducted at Evotec (UK) Ltd (Manchester, UK). Measurement of activity  
177 against other fungi was performed by Eurofins Scientific according to methodology described by  
178 the Clinical and Laboratory Standards Institute (CLSI) (CLSI M38-A (19) or M27-A2 (20),  
179 www.eurofinspanlabs.com).

180

181 ***In vitro* determination of persistence of action on *A. fumigatus* hyphae.** The persistence of  
182 action of test agents was calculated in *A. fumigatus* hyphae (NCPF2010). Conidia were diluted in  
183 growth media (RPMI-1640, 2 mM L-glutamine, 2 % glucose, 0.165 M MOPS, pH 7.0) and  
184 added across a 384-well plate at a final concentration of  $1 \times 10^3$ /well. After incubation at 35°C for  
185 exactly 6 h, test and reference articles or neat DMSO (as vehicle) (0.5  $\mu$ l /well) were added to the  
186 appropriate wells to give a final concentration of 0.5% DMSO. The plates were incubated for  
187 exactly 20 minutes at 35°C and 5% CO<sub>2</sub>. After the incubation time had elapsed all wells on the  
188 designated washout plate were aspirated and growth media (100  $\mu$ l/well) was added across the  
189 plate. For the non-washout plate, after compounds were added to hyphae, no media change was  
190 applied. Resazurin (0.04% diluted in growth media) was added to all wells of both non-washout

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191 and washout plates (5  $\mu$ l/well) to give a final concentration of 0.002% resazurin. The plates were  
192 incubated at 35°C and 5% CO<sub>2</sub> for 16 h. Subsequently fluorescence in each well was measured at  
193  $\lambda_{ex}/\lambda_{em}$  545/600 nm using a multiscanner (Clariostar: BMG, Buckinghamshire, UK). The  
194 percentage inhibition for each well was calculated and the IC<sub>50</sub> value was calculated from the  
195 concentration-response curve generated for each test compound using a four-parameter logistic  
196 equation (Dotmatics, Bishops Stortford, UK).

197

198 ***In vitro* determination of persistence of action on bronchial epithelial cells.** The persistence  
199 of action of test agents was evaluated in immortalised, bronchial, epithelial cells (BEAS2B).  
200 Each experiment consisted of one non-washout plate (96-well) and a parallel washout plate into  
201 which BEAS2Bs were seeded at a concentration of 3x10<sup>4</sup> cells/well in growth media (RPMI-  
202 1640, 2 mM L-glutamine, 10% FCS), and incubated for 24 h at 37°C, 5% CO<sub>2</sub>. Test and  
203 reference articles or neat DMSO (as vehicle) (0.5  $\mu$ l /well) were added to the appropriate wells of  
204 the washout plate to give a final concentration of 0.5% DMSO. The plate was incubated for  
205 exactly 1 h at 37°C and 5% CO<sub>2</sub>. After the incubation time had elapsed all wells on the washout  
206 plate were aspirated and growth media (100  $\mu$ l/well) was added across the plate. After 24 h  
207 incubation at 37°C, test and reference articles or neat DMSO (as vehicle) (0.5  $\mu$ l /well) were  
208 added to the appropriate wells of the non-washout plate to give a final concentration of 0.5%  
209 DMSO. The plate was incubated for exactly 1 h at 37°C and 5% CO<sub>2</sub> after which *A. fumigatus*  
210 conidia were added across both plates at a final concentration of 1 x 10<sup>3</sup>/well. Fungal growth was  
211 determined after a further 24 h incubation at 35°C, 5% CO<sub>2</sub>, by measuring galactomannan (GM)  
212 concentrations, using Platelia GM-EIA kits (Bio-Rad Laboratories, 62794). The percentage  
213 inhibition for each well was calculated and the IC<sub>50</sub> value was calculated from the concentration-

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214 response curve generated for each test compound using a four-parameter logistic equation  
215 (Dotmatics, Bishops Stortford, UK).

216

217 ***In vivo* antifungal activity against *A. fumigatus* infection.** Specific pathogen-free A/J mice  
218 (male, 5 weeks old) were purchased from Sankyo Labs Service Co. Ltd. (Tokyo, Japan) and  
219 adapted for 1 week in a temperature ( $24 \pm 1^\circ\text{C}$ ) and humidity ( $55 \pm 5\%$ ) controlled room, under a  
220 12 h day-night cycle. The mice were reared on a standard diet and tap water *ad libitum*. A/J mice  
221 were used for *A. fumigatus* infection and proved to be more efficiently infected as described  
222 previously (21). Animals were then dosed with hydrocortisone (Sigma H4881, 125 mg/kg,  
223 subcutaneously) on days 3, 2 and 1 before infection, and with cyclophosphamide (Sigma C0768;  
224 250 mg/kg, intraperitoneally) two days before infection to induce temporary neutropenia as  
225 previously reported (22). To avoid bacterial infection, drinking water was supplemented with  
226 tetracycline hydrochloride (Sigma T7660; 1  $\mu\text{g/ml}$ ) and ciprofloxacin (Fluka 17850; 64  $\mu\text{g/ml}$ ).  
227 *A. fumigatus* (ATCC13073 [strain: NIH 5233], purchased from the American Type Culture  
228 Collection, Manassas, VA, USA) was grown on malt agar (Nissui Pharmaceutical, Tokyo, Japan)  
229 plates for 6–7 days at room temperature ( $24 \pm 1^\circ\text{C}$ ). Conidia were aseptically dislodged from the  
230 agar plates and suspended in sterile distilled water with 0.05% Tween 80 and 0.1% agar. On the  
231 day of infection, conidial counts were assessed by haemocytometer and the inoculum was  
232 adjusted to obtain a concentration of  $1.67 \times 10^8/\text{ml}$  in physiological saline. On day 0, 30  $\mu\text{l}$  of the  
233 conidia suspension was administered intranasally.

234 Test agents, suspended in physiological saline, were administered daily intranasally (35  $\mu\text{l}$ )  
235 on days 1 to 6 and the survival of animals was recorded for 7 days. The volume inserted  
236 intranasally is reported to achieve almost 60% deposition into the lung (23). Deaths and the body  
237 weights of surviving animals were monitored daily. A body weight loss of  $> 20\%$ , compared

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238 with an animal's weight on day 1, or a mouse death, were both defined as "drop-out" events.  
239 Animals that lost > 20% of their initial body weight were sacrificed. It was observed that *A.*  
240 *fumigatus* infection induced a "rolling" behaviour, which was monitored and recorded (24).  
241 Bronchoalveolar lavage fluid (BALF) was collected on day 7 post infection or on the day that the  
242 mouse dropped out of the study. The *Aspergillus* GM concentration in BALF was determined  
243 with Platelia GM-EIA kits (Bio-Rad Laboratories, 62794). The value was provided as a "cut-off  
244 index" (COI) which was calculated by the formula:  $COI = OD \text{ in sample} / OD \text{ in cut-off control}$ ,  
245 provided by the kit. All animal studies were approved by the Ethics Review Committee for  
246 Animal Experimentation of Nihon University. *A. fumigatus* studies were approved by the  
247 Microbial Safety Management Committee of Nihon University School of Pharmacy (E-H25-  
248 001).  
249  
250 **Statistical analysis** Results are expressed as means  $\pm$  standard error of the mean (SEM).  
251 Survival analysis was performed by Kaplan-Meier plots followed by the log rank (Mantel-Cox)  
252 tests using the PRISM 6<sup>®</sup> software program (GraphPad Software Inc., San Diego, CA, USA).  
253 For comparison between groups either the ordinary one-way ANOVA with Tukey's *post hoc*  
254 comparison, or the Kruskal-Wallis ANOVA with Dunn's *post hoc* comparison was performed.  
255 Statistical significance was defined as  $P < 0.05$ .  
256

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257 **RESULTS**

258 **CYP51 binding properties.** PC945 produced a type II difference spectrum when titrated against  
259 purified *A. fumigatus* CYP51A and CYP51B enzymes and bound with a similar affinity to  
260 CYP51A as posaconazole (Table 1; Fig. 1B). In contrast, in ligand titration experiments with  
261 purified CYP51B, PC945 yielded a sigmoid binding saturation curve whilst posaconazole gave  
262 the expected tight binding saturation curve (Fig. S1). A modified two site allosteric model gave  
263 the best 'off-the-shelf' fit of the sigmoid PC945 saturation curve yielding  $K_d1$  and  $K_d2$  values of  
264 19298  $\mu\text{M}$  and 0.32  $\mu\text{M}$ . This positive cooperative allosterism suggests either the existence of  
265 two non-equivalent ligand binding sites or the existence of two different binding conformations /  
266 orientations for the PC945 molecule within CYP51B that are responsible for the generation of  
267 the type II difference spectrum. Posaconazole bound tightly to purified CYP51B with a  $K_d$  value  
268 of 0.012  $\mu\text{M}$ .

269

270 **Inhibitory activity against *A. fumigatus* CYP51 enzyme.** The inhibitory activity of PC945 and  
271 posaconazole against *A. fumigatus* sterol 14 $\alpha$ -demethylases were determined using 0.5  $\mu\text{M}$  *A.*  
272 *fumigatus* CYP51A and 0.5  $\mu\text{M}$  CYP51B in the membrane fraction prepared from *E. coli*  
273 expression clones. Both PC945 and posaconazole were strong tight-binding inhibitors of  
274 CYP51A and CYP51B *in vitro* activity (Table 1; Fig. 1C), suggesting  $K_{i,app}$  values of below 1  
275 nM for both compounds (25). Moreover, PC945 was equally effective as posaconazole, and both  
276 agents appear to share the same mode of action; by directly coordinating as the sixth axial ligand  
277 of the CYP51 heme iron. No allosterism was observed during the inhibition of AfCYP51B  
278 activity by PC945.

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280 **Cell based *A. fumigatus* sterol composition and CYP51 assay.** Analysis of sterol composition  
281 was performed by GC-MS. Treatment with increasing concentrations of either posaconazole or  
282 PC945 from 0 to 1  $\mu\text{g ml}^{-1}$  resulted in the dose dependent accumulation of the 14  $\alpha$ -methylated  
283 sterols (lanosterol and eburicol) and the corresponding depletion of the final sterol product:  
284 ergosterol (Table 2, Fig. 1D).

285 We also investigated enzyme inhibitory activity in a plate-based *A. fumigatus* cell-based  
286 ergosterol assay. This test system takes advantage of the fact that cholesterol oxidase can utilise  
287 ergosterol as a substrate with a 65% loss of sensitivity. Oxidation of ergosterol was determined  
288 by observing the conversion of the weakly fluorescent resazurin to the highly red fluorescent  
289 resorufin and was normalised using crystal violet staining. Resembling its inhibitor activity in the  
290 cell-free model of CYP51, PC945 strongly inhibited ergosterol production ( $\text{IC}_{50} = 0.0047 \mu\text{g/ml}$ ;  
291  $0.0069 \mu\text{M}$ ) and was 14 and 2.6-fold more potent than voriconazole ( $\text{IC}_{50} = 0.067 \mu\text{g/ml}$ ;  $0.19$   
292  $\mu\text{M}$ ) and posaconazole ( $\text{IC}_{50} = 0.012 \mu\text{g/ml}$ ;  $0.017 \mu\text{M}$ ), respectively.

293  
294 ***In vitro* antifungal activity against azole susceptible and azole resistant strains of *A.***  
295 ***fumigatus*.** The concentrations of test agents required to achieve 50% inhibition ( $\text{IC}_{50}$  [OD]) and  
296 90% inhibition ( $\text{IC}_{90}$  [OD]) of the growth of a number of *A. fumigatus* strains (Itraconazole  
297 susceptible-NCPF2010, AF294, AF293; Itraconazole resistant-AF72, AF91, TR34-L98H), were  
298 calculated from growth curves generated using a modified 384-well EUCAST microdilution  
299 method and compared to positive and negative controls. Overall, PC945 was more active than all  
300 reference compounds including voriconazole, posaconazole, and itraconazole, against  
301 itraconazole-susceptible *A. fumigatus* strains (26, 27) (NCPF2010, AF294, AF293, Table 3). In  
302 addition, PC945 was the most active test agent against known itraconazole-resistant *A. fumigatus*  
303 strains (AF72, AF91) (28, 29) (Table 3). Against the *A. fumigatus* strain L98H, containing the

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304 environmentally acquired TR34/L98H mutation (30), PC945, voriconazole, itraconazole, and  
305 caspofungin all failed to achieve 90% inhibition of fungal growth, whilst posaconazole displayed  
306 an IC<sub>90</sub> value of 0.13 µg/ml. However, PC945 achieved an IC<sub>50</sub> value of 0.034 µg/ml against this  
307 strain, thereby revealing it to be 2.5-fold more potent than posaconazole (Table 3). BSA  
308 supplementation was confirmed to have no or little effect on the MIC of voriconazole (2 µg/ml  
309 without BSA, 1-2 µg/ml with BSA), posaconazole (0.03 µg/ml without BSA, 0.06 µg/ml with  
310 BSA), itraconazole (0.25 µg/ml without BSA, 0.125 µg/ml with BSA) and amphotericin B (1  
311 µg/ml without BSA, 2 µg/ml with BSA) using *A. fumigatus* quality control strain ATCC204305,  
312 but it showed marginal effects on the MIC of PC945, a more lipophilic compound (0.25 µg/ml  
313 without BSA, 0.0625 µg/ml with BSA).

314

315 ***In vitro* antifungal activity against clinically isolated *A. fumigatus*.** Test agents were evaluated  
316 in 50 clinical isolates obtained from the Saint Louis Hospital (Paris, France) and 46 clinical  
317 isolates obtained from the NW Mycology Centre in the UK. PC945 was found to be 2.5 fold  
318 more potent than voriconazole but comparable to posaconazole based on the geometric mean  
319 (Table 4). Among the clinical isolates from the NW Mycology Centre, 13 of the 46 strains were  
320 found to be posaconazole resistant, 7 of 13 posaconazole resistant isolates were confirmed as  
321 itraconazole resistant, and two of them were pan-azole resistant including voriconazole, based on  
322 the EUCAST ECOFF. In five of the posaconazole resistant isolates, PC945 did not inhibit  
323 growth completely at concentrations up to 8 µg/ml. During this assay, the quality control strain  
324 *A. fumigatus* ATCC204305 was used for validation. In this strain posaconazole showed a MIC of  
325 0.25 µg/mL, within the range set by the EUCAST guidelines.

326



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327 ***In vitro* assessment of antifungal activity using CLSI methodology.** Visual assessment of the  
328 growth of four itraconazole susceptible *A. fumigatus* strains demonstrated that PC945 was the  
329 most potent compound tested with an MIC value of 0.031 µg/ml; whilst voriconazole and  
330 posaconazole were less effective (Table 5). Thus the superiority of PC945 to voriconazole on *A.*  
331 *fumigatus* growth inhibition was confirmed by the CLSI method as well as the EUCAST  
332 microdilution method.

333

334 **Antifungal activity against non-*A. fumigatus* species.** The *in vitro* activities of PC945,  
335 voriconazole, and posaconazole against 22 pathogenic fungi (1~2 isolates each) are displayed in  
336 Table 6. The data for *A. terreus* shows it to be more susceptible to PC945 than posaconazole. In  
337 addition, PC945 was found to have antifungal activity against *A. carbonarius* and *A. flavus*,  
338 albeit less potently than posaconazole or voriconazole. Against *C. albicans*, (both azole-  
339 susceptible and azole-resistant strains), *C. glabrata*, and *C. krusei*, PC945 was generally more  
340 active than voriconazole and equally potent to posaconazole. PC945, voriconazole and  
341 posaconazole were comparable in effectiveness against *T. rubrum*. The remarkable potency of  
342 PC945 against *R. oryzae* was seen in a greatly improved MIC (2 µg/ml) when compared to  
343 voriconazole and posaconazole (MIC, > 8 µg/ml). The potency of PC945 against *C. neoformans*  
344 and *C. gattii* was higher than or similar to voriconazole and posaconazole. In contrast, *A. niger*,  
345 *A. pullulans*, *P. chrysogenum*, *P. citrinum*, *C. argillaceum*, *C. globosum*, *G. zae* (*Fusarium*  
346 *graminearum*), *L. corymbifera*, *M. circinelloides*, and *R. pusillus* were not susceptible to PC945  
347 treatment within the concentration range tested.

348

349 ***In vitro* determination of persistence of action.** Retention of test agents within the hyphae of *A.*  
350 *fumigatus* was determined using a resazurin-based microtitre assay. The inhibition of fungal

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351 growth, arising from continuous contact of the test compounds with *A. fumigatus* hyphae for 16  
352 h, was measured and compared with that obtained after contact with drug for only 20 minutes,  
353 followed by washout and incubation for the same period. As seen in Table 7 and Fig. 2B, the  
354 antifungal potency of voriconazole and posaconazole diminished markedly, after short contact  
355 and washout, by a factor of > 93-fold and 4.9-fold, respectively. In contrast, PC945 showed no  
356 change in its antifungal activity between wash and non-wash in this experimental paradigm  
357 (Table 7). It was also observed that PC945 (IC<sub>50</sub>: 0.00010 µg/ml) was 110 and 4.5-fold more  
358 potent, than either voriconazole or posaconazole at inhibiting hyphal *A. fumigatus* growth.

359 In the second system, the persistence of action of PC945 was quantified using GM  
360 production in supernatant as an index of fungal growth. BEAS2B cells were infected with *A.*  
361 *fumigatus*, and the effects of a 24 h washout period prior to infection were examined. A one hour  
362 contact time with PC945 followed by 24 h washout resulted in an approximate 11-fold loss of  
363 potency against *A. fumigatus*, compared with the control in which there was no washout.  
364 Although posaconazole showed a similar or slightly greater loss of potency on washout, it was  
365 notable that voriconazole was ineffective after a 24 h washout. These data imply that only a short  
366 contact period of bronchial epithelial cells with PC945 would be required for the agent to exhibit  
367 a long duration of therapeutic action (Table 7, Fig. 2C).

368

369 ***In vivo* antifungal activity.** The potential of intranasally administered PC945 as a daily  
370 therapeutic treatment for pulmonary *Aspergillus* infection was investigated using  
371 immunocompromised, temporarily neutropenic mice. In this model, 81% of control mice (13/16)  
372 were dead or had dropped out by day 6 post infection and only 19% of mice survived (Table 8,  
373 Fig. 2D). However, it was observed that 50, 63 and 63% of mice dosed with PC945 (0.56, 2.8  
374 and 14 µg/mouse: intranasal application of 0.016, 0.08 and 0.4 mg/ml suspension) survived

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375 (Table 8, Fig. 2D). In contrast, although the highest dose (70 µg/mouse) showed a 75% survival  
376 rate, the effects of posaconazole at 2.8 and 14 µg/mouse were weaker than that of PC945 at 0.56  
377 µg/mouse (Table 8, Fig. 2E). Furthermore, GM in BALF collected on day 7 post infection, or on  
378 the day that an animal dropped out of the study, was significantly reduced by treatment with  
379 PC945 to a superior degree than obtained with posaconazole treatment (Fig. 2F). Intranasal *A.*  
380 *fumigatus* infection is known to cause “rolling” behaviour in mice due to CNS effects via  
381 respiratory-systemic infection (24). In this study it was noted that PC945, but not posaconazole,  
382 substantially inhibited the incidence of this rolling behaviour (supplement Fig. 2).

383

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384 **DISCUSSION**

385 It has been demonstrated herein that PC945 is a potent antifungal agent, possessing  
386 activity against a broad range of both azole-susceptible and azole-resistant strains of *A.*  
387 *fumigatus*. Against itraconazole-susceptible *A. fumigatus* strains, PC945 showed an increase in  
388 potency between 20 and 180-fold over voriconazole, and comparable or improved potency  
389 versus posaconazole. In an itraconazole-resistant strain of *A. fumigatus* with a CYP51A M220V  
390 mutation, obtained from a patient undergoing high-dose itraconazole therapy, and an *A.*  
391 *fumigatus* strain harbouring the G54E mutation, recently discovered to be environmentally  
392 acquired (26, 31), PC945 was 6 and 2-fold more potent than voriconazole and posaconazole,  
393 respectively. Furthermore, in a strain of *A. fumigatus*, isolated in France (30) and displaying an  
394 environmentally acquired TR34/L98H mutation, PC945 was more active than voriconazole,  
395 although it did not achieve 90% inhibition of growth.

396 Against 96 clinically isolated *A. fumigatus* strains, obtained from St. Louis Hospital, Paris  
397 and the North West Mycology Centre in the UK (Evotec UK Ltd), the MIC range for PC945 was  
398 found to be between 0.032 and 16 µg/ml, with a geometric mean of 0.17 µg/ml and the MIC<sub>50</sub>  
399 and MIC<sub>90</sub> values were 0.125 and 1.0 µg/ml, respectively. The potency of PC945 was superior to  
400 voriconazole and comparable to posaconazole. It is noteworthy that out of 46 clinical isolates of  
401 *A. fumigatus* from the NW Mycology Centre, 13 strains were posaconazole resistant and two of  
402 them were pan-azole resistant including voriconazole, based on the EUCAST ECOFF. Several  
403 reports have demonstrated that there is an increasing incidence of itraconazole resistant *A.*  
404 *fumigatus* in the north-west due to extensive clinical use of itraconazole in this area of the UK  
405 (32, 33). PC945 showed inhibitory activity against 8 of the 13 azole resistant strains, but did not  
406 inhibit the growth of five strains completely when used at concentrations up to 16 µg/ml.

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407 Although clearly resistant to azoles, the genetic cause(s) underlying resistance in these strains is  
408 unknown.

409 In this study we modified the original EUCAST system. Firstly, for our in-house  
410 screening regimen we adapted the EUCAST methodology to a 384-well system, as this is a more  
411 efficient assay which has been shown in other fungal species to generate comparable data to the  
412 96-well format assay (34). The same final concentration of DMSO was applied across the plate  
413 to compare treatment effects with vehicle control more accurately: currently EUCAST suggests  
414 dilution of compounds with EUCAST media, but some lipophilic or insoluble compounds may  
415 precipitate generating misleading data. In fact, PC945 precipitated in EUCAST media at >  
416 4µg/ml, but voriconazole (less lipophilic) did not. Furthermore, data from Pulmocide and from  
417 the St Louis Hospital in Paris were generated by supplementing growth media with 0.5% BSA,  
418 as this avoids the loss of lipophilic compounds bound to plastic surfaces during the assay and is  
419 not inhibitory to *A. fumigatus* growth (35, 36). Using the quality control *A. fumigatus* strain  
420 ATCC204305, we confirmed BSA supplementation did not affect the MIC of known antifungal  
421 agents including voriconazole, posaconazole, itraconazole and amphotericin B. Lastly, at  
422 Pulmocide we used turbidity, determined by assessment of OD using a spectrophotometer, as a  
423 measure of fungal growth to enable more accurate quantification of the inhibitory effects of  
424 treatments. All the modifications above helped to quantify the antifungal activity of test agents  
425 more accurately.

426 Whilst *A. fumigatus* represents a severe, global, health risk, other fungal species continue  
427 to be equally problematic. Invasive candidiasis affects 46,000 people each year in the US alone  
428 and an estimated 1 million people with HIV/AIDS contract cryptococcal meningitis worldwide  
429 annually (37, 38). These figures underline the pressing need for safer and more effective  
430 antifungals that deliver a broad spectrum of activity. This report discloses that the novel agent

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431 PC945 has a broad activity profile against a diverse range of fungal species. The growth of *C.*  
432 *albicans*, *C. glabrata*, and *C. krusei*, was inhibited by PC945 as strongly as with posaconazole  
433 and 1.7-16 fold more actively than with voriconazole. Against *C. neoformans* PC945 was two-  
434 fold more potent than both voriconazole and posaconazole, whilst *C. gattii* was equally  
435 susceptible to the inhibitory activities of PC945, voriconazole and posaconazole. Mucormycosis  
436 caused by *R. oryzae* has a mortality rate of 76% for patients with pulmonary infections (39). In  
437 this study PC945 was particularly effective against *R. oryzae* (MIC, value of 2.0 µg/ml) whilst  
438 voriconazole and posaconazole had no effect within the concentration range tested.

439 The proposed mechanism of action of PC945 is inhibition of sterol 14 $\alpha$ -demethylase  
440 (CYP51A1), the enzyme required to convert eburicol to 14-demethylated eburicol, an essential  
441 step in the ergosterol biosynthesis pathway in fungi. Type II binding spectra, which display an  
442  $A_{\max}$  at 423-430 nm and a broad trough at 386-412 nm arise through a specific interaction in  
443 which the triazole *N*-4 nitrogen (posaconazole) or the imadazole ring *N*-3 nitrogen coordinates as  
444 the sixth axial ligand with the heme iron to form a low-spin CYP51-azole complex (40, 41).  
445 PC945 produced type II difference spectra when titrated against purified *A. fumigatus* CYP51A  
446 and CYP51B enzymes, and bound with a similar affinity to CYP51A as posaconazole, but  
447 yielded a sigmoid binding saturation curve against CYP51B. This latter binding characteristic of  
448 PC945 was not reflected in the compound's inhibition of CYP51B activity, suggesting a  
449 difference in the enzyme's conformation in solution and that adopted when incorporated in cell  
450 membranes. Furthermore, the strong inhibition of CYP51A activity observed with both PC945  
451 and posaconazole, indicative of tight-binding inhibitors (IC<sub>50</sub> value approximately half that of the  
452 enzyme concentration present) was tighter than predicted by the calculated  $K_d$  values from ligand  
453 binding studies using recombinant CYP51A, suggesting the conformation of purified CYP51A in  
454 solution differs to that in cell membranes. In the sterol composition assay, treatment with

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455 increasing concentrations of either PC945 or posaconazole, from 0 to 1  $\mu\text{g ml}^{-1}$ , resulted in an  
456 accumulation of the 14-methylated sterols, lanosterol and eburicol, and depletion of the final  
457 sterol product, ergosterol; this being consistent with CYP51 inhibition as the key  
458 pharmacological activity of both agents. In addition, the *A. fumigatus* cell-based ergosterol assay  
459 demonstrated that PC945 was 14 and 2.6-fold more potent at inhibiting ergosterol production  
460 than voriconazole and posaconazole, respectively. Thus, the mechanism of action of PC945, as  
461 for other triazole antifungals, is the inhibition of fungal sterol 14 $\alpha$ -demethylase; resulting in the  
462 depletion of ergosterol in the fungal membrane; thereby disrupting membrane structure and  
463 function and inhibiting growth of the pathogenic organism (14).

464 A highly desirable feature of topical medicines is a long duration of action ensuring that  
465 the desired therapeutic activity is maintained throughout the inter-dose period. In order to  
466 explore this parameter the persistence of action of PC945 in a number of *in vitro* systems was  
467 studied. In *A. fumigatus* hyphae the IC<sub>50</sub> value measured for PC945 following a 20 minute  
468 contact period and washout for 16 h was almost unchanged relative to that obtained following  
469 continuous contact with the drug for the same period without washout. Furthermore, in the  
470 BEAS2B cell line, washout for 24 h, after a 1 h contact period, resulted in only an approximate  
471 10-fold loss of potency against *A. fumigatus* compared with control in which there was no  
472 washout period. This property of cellular persistence, in the absence of the pathogen, may be a  
473 particularly valuable property by enhancing the potential use of PC945 in prophylaxis.

474 The anti-*Aspergillus* activity of PC945, administered intranasally, was also investigated  
475 in mice using a survival read-out. PC945 was dosed to animals, pre-treated with a single round of  
476 cyclophosphamide and three rounds of hydrocortisone to induce temporary neutropenia;  
477 followed 24 h later by inoculation with *A. fumigatus*. In this study 81% of vehicle treated, *A.*  
478 *fumigatus* infected mice were classed as dead/dropout by day seven. However, once-daily

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479 treatment with low dose PC945 showed marked beneficial effects on survival. Whilst 44% of  
480 infected mice survived to day 7 when treated with posaconazole at 14  $\mu\text{g}/\text{mouse}$ , those treated  
481 with PC945 showed 50% survival at a 25-fold lower dose (0.56  $\mu\text{g}/\text{mouse}$ ). Despite displaying  
482 similar antifungal activities as determined by the broth microdilution assay, these results indicate  
483 that PC945 significantly outperforms posaconazole *in vivo*. This superior profile probably arises  
484 from two factors, the first pharmacokinetic and the second pharmacodynamic. As discussed  
485 earlier, PC945 exhibits a longer duration of action than posaconazole and the molecule is  
486 retained within the lung, such that little systemic exposure results, compared with posaconazole,  
487 after intranasal treatment (unpublished data). As a clinical strategy, topical treatment of the lung  
488 is advantageous over oral or intravenous therapy as it delivers high concentrations of an  
489 antifungal agent directly to the site of infection and avoids unfavourable systemic side-effects.  
490 The benefits of inhaled administration for the treatment of invasive pulmonary aspergillosis has  
491 been shown in numerous studies involving amphotericin B, itraconazole and voriconazole (42-  
492 44).

493 Development of resistance to antifungal therapy is an increasing problem in recent years.  
494 It has been shown that a strategy for avoiding the development of resistance is to ensure that the  
495 ratio of treatment peak concentration to MIC is adequate (45). The relationship between  
496 resistance mutation induction and drug exposure has been well-studied in bacteria. In the case of  
497 levofloxacin use against infection with *Pseudomonas aeruginosa*, exposure at an AUC/MIC ratio  
498 of 157 was calculated to prevent emergence of resistance (46). In the same manner, delivery of  
499 antifungals directly to the lung enables high AUC/MIC ratios to be achieved locally, reducing the  
500 risk of resistance occurring.

501 PC945 is the first antifungal specifically designed as a once-daily, topical, inhaled  
502 treatment for *Aspergillus* infection of the lung. Designed to be retained within the target organ



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503 (such as lung), treatment with PC945 results in very low systemic exposure (data not shown)  
504 thus reducing the potential risk for unwanted clinical effects. In addition, PC945 exhibits high  
505 levels of plasma protein binding, further reducing the likelihood of problems arising from  
506 circulating drug substance. Therefore, PC945 has the pharmacological and pharmaceutical  
507 properties to be a best-in-class, inhaled therapy for the treatment of *A. fumigatus* infection in  
508 man.

509

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661 **FIGURE LEGEND**

662 **FIGURE 1 Efficacy of PC945 as an inhibitor of *A. fumigatus* sterol 14 $\alpha$ -demethylase**  
663 **(CYP51).** (a) Structure of PC945, (b) Type II azole binding spectra for *A. fumigatus* CYP51A  
664 and CYP51B, each experiment was performed 4-6 times although only one replicate is shown (c)  
665 Azole IC<sub>50</sub> determinations of posaconazole (●) and PC945 (○), mean relative velocity values  
666 are shown with standard deviations, (d) Sterol composition of *A. fumigatus* treated with PC945,  
667 the relative levels of lanosterol and eburicol.

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669 **FIGURE 2 Antifungal activity of PC945 against *A. fumigatus* *in vitro* and *in vivo*.**

670 (a) A549 treated with PC945 or posaconazole and infected with GFP-*A. fumigatus*: green = GFP-  
671 *A. fumigatus*; Blue = DAPI-stained nucleus; Red = Celltracker-stained cytoplasm, (b) Persistence  
672 of action of PC945 and voriconazole on *A. fumigatus* hyphae, (c) Persistence of action of PC945  
673 and voriconazole on human bronchial cell lines (Beas2B) infected with *A. fumigatus*, (d, e)  
674 Effect of once daily intranasal treatment of PC945 (0.56, 2.8, 14  $\mu$ g/mouse) and posaconazole  
675 (2.8, 14, 70  $\mu$ g/mouse) on survival in *A. fumigatus* infected immunocompromised mice  
676 (N=8~16), (f) Galactomannan in BALF, each horizontal bar was presented as mean  $\pm$  SEM from  
677 8 mice per group. # Dead before day 7. \*\*\* Significant difference from no infection at  $P<0.001$ .  
678 ††† Significant difference from infection control at  $P<0.001$ .

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684 **TABLE 1** Azole  $K_d$  and  $IC_{50}$  determinations versus *A. fumigatus* CYP51 (AfCYP51) enzymes

Test Agent	AfCYP51A		AfCYP51B		
	$K_d$ ( $\mu$ M) <sup>a</sup>	$IC_{50}$ ( $\mu$ M) <sup>b</sup>	$K_d1$ ( $\mu$ M) <sup>a</sup>	$K_d2$ ( $\mu$ M) <sup>a</sup>	$IC_{50}$ ( $\mu$ M) <sup>b</sup>
<b>PC945</b>	0.50	0.23	19298	0.32	0.22
<b>Posaconazole</b>	0.96	0.16	0.012	n/a	0.17

685 <sup>a</sup>  $K_d$  determinations used 4  $\mu$ M purified AfCYP51A and 4  $\mu$ M AfCYP51B proteins.

686 <sup>b</sup>  $IC_{50}$  determinations used 0.5  $\mu$ M AfCYP51A and 0.5  $\mu$ M AfCYP51B recovered in the  
687 membrane fraction from the *E. coli* expression clones.

688 n/a = not applicable

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705 **TABLE 2** % Sterol composition of *A. fumigatus* treated with either posaconazole or PC945

Sterol	Sterol compositions (posaconazole-treated [ $\mu\text{g/ml}$ ])					
	DMSO	0.0001	0.001	0.01	0.1	1
Ergosterol	100	94.5	87.2	74.7	67.8	67.4
Ergost-5,7-dienol	0	3.3	3.9	0	0	0
Lanosterol	0	0	3.0	7.0	8.8	8.8
Eburicol	0	2.2	5.9	18.3	23.4	23.8

Sterol	Sterol compositions (PC945-treated [ $\mu\text{g/ml}$ ])					
	DMSO	0.0001	0.001	0.01	0.1	1
Ergosterol	100	95.9	94.7	86.7	80.6	71.3
Ergost-5,7-dienol	0	4.1	2.5	3.3	0	0
Lanosterol	0	0	0	3.6	6.2	9.4
Eburicol	0	0	2.7	6.5	13.2	19.3

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720 **TABLE 3** Antifungal effects of PC945 and known antifungal agents in azole susceptible and azole resistant strains of *A. fumigatus*<sup>a</sup>

Strain Number	Resistance Mechanism	IC <sub>50</sub> and (IC <sub>90</sub> ) Values for Agent Indicated (µg/ml)					
		PC945	Voriconazole	Posaconazole	Itraconazole	Amphotericin B	Caspofungin
NCPF2010	none	<b>0.0084</b> (0.010)	<b>0.16</b> (0.20)	<b>0.0086</b> (0.014)	<b>0.057</b> (0.085)	<b>0.23</b> (0.48)	<b>0.11</b> (>1)
AF294	none	<b>0.0020</b> (0.0043)	<b>0.082</b> (0.27)	<b>0.0056</b> (0.011)	<b>0.041</b> (0.052)	<b>0.21</b> (0.79)	>1 (>1)
AF293	none	<b>0.0012</b> (0.0041)	<b>0.25</b> (0.74)	<b>0.010</b> (0.028)	<b>0.032</b> (0.23)	<b>0.24</b> (0.85)	>1 (>1)
AF72	G54E	<b>0.0061</b> (0.029)	<b>0.019</b> (0.062)	<b>0.032</b> (0.19)	<b>0.43</b> (>1)	<b>0.18</b> (0.64)	<b>0.10</b> (>1)
AF91	M220V	<b>0.0081</b> (0.059)	<b>0.12</b> (0.38)	<b>0.024</b> (0.12)	<b>0.26</b> (>1)	<b>0.42</b> (>1)	<b>0.072</b> (>1)
L98H	TR34/L98H	<b>0.034</b> (>1)	>1 (>1)	<b>0.086</b> (0.13)	<b>0.22</b> (>1)	<b>0.14</b> (0.29)	<b>0.082</b> (>1)

721 <sup>a</sup> IC<sub>50</sub> and IC<sub>90</sub> values were determined from optical density measurements

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731 **TABLE 4** *In vitro* activities of PC945, posaconazole and voriconazole against 96 clinically  
732 isolated *A. fumigatus* strains <sup>a</sup>

Test Agent	MIC (µg/ml)				
	MIC [range]	Geo-mean <sup>b</sup>	Mode	MIC <sub>50</sub>	MIC <sub>90</sub>
<b>PC945</b>	0.032->8	0.17 <sup>*</sup>	0.125	0.125	1
<b>Voriconazole</b>	0.064-4	0.42	0.5	0.5	1
<b>Posaconazole</b>	0.016-2	0.1	0.032	0.063	0.5

733 <sup>a</sup> All MIC were determined visually; MIC<sub>50</sub> and MIC<sub>90</sub> values represent the concentrations  
734 required to inhibit 50 and 90% of the strains tested.

735 <sup>b</sup> Geo-mean = geometric mean.

736 <sup>\*</sup> P < 0.05; PC945 versus the results for posaconazole (One way ANOVA with Tukey's test).

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751 **TABLE 5** Antifungal effect of PC945 and known antifungal agents on 4 itraconazole susceptible  
752 *A. fumigatus* strains (CLSI methodology)

Test Agent	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>		MIC <sub>50</sub> ( $\mu\text{g/ml}$ ) <sup>b</sup>	
	Median	Interquartile	Median	Interquartile
<b>PC945</b>	0.031*	0.020–0.031	0.011**	0.0083–0.024
<b>Voriconazole</b>	0.5	0.5–0.5	0.14	0.082–0.15
<b>Posaconazole</b>	0.047	0.031–6.0	0.015	0.0095–0.016

753 <sup>a</sup> MIC determined visually.

754 <sup>b</sup> MIC<sub>50</sub> determined using optical density measurements.

755 \* P < 0.05, \*\* P < 0.01; PC945 versus the results for voriconazole (Kruskal-Wallis one-way  
756 ANOVA with Dunn's test).

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771 **TABLE 6** Antifungal effects of PC945 and posaconazole on other fungal species

Species (Strain/s)	Strains tested	Culture method	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>		
			PC945	Voriconazole	Posaconazole
<i>Aspergillus carbonarius</i> (ATCC8740)	1	CLSI	4	0.5	0.063
<i>Aspergillus flavus</i> (ATCC204304)	1	CLSI	>8	2	0.13
<i>Aspergillus flavus</i> (AFL8; NRRC3357)	2	EUCAST	6	0.63	0.16
<i>Aspergillus niger</i> (ATCC1015)	1	EUCAST	>8	1	0.20
<i>Aspergillus terreus</i> (AT49; AT7130)	2	EUCAST	0.078	1	0.093
<i>Penicillium chrysogenum</i> (ATCC9480)	1	CLSI	>8	2	0.13
<i>Penicillium citrinum</i> (ATCC9849)	1	CLSI	>8	>8	0.5
<i>Trichophyton rubrum</i> (ATCC10218)	1	CLSI	0.031	0.063	0.031
<i>Aureobasidium pullulans</i> (ATCC9348)	1	CLSI	>8	>8	1
<i>Cladosporium argillaceum</i> (ATCC38013)	1	CLSI	>8	0.5	0.25
<i>Candida albicans</i> <sup>b</sup> (20240.047; ATCC10231)	2	CLSI	0.081	0.14	0.081
<i>Candida albicans-AR</i> <sup>b</sup> (20183.073; 20186.025)	2	CLSI	8.25	10	8.13
<i>Candida glabrata</i> <sup>b</sup> (ATCC36583; R363)	2	CLSI	0.5	8.13	0.5
<i>Candida krusei</i> (ATCC6258)	1	CLSI	0.125	0.25	0.125
<i>Chaetomium globosum</i> (ATCC44699)	1	CLSI	>8	1	0.25
<i>Gibberella zeae</i> ( <i>Fusarium graminearum</i> ) (ATCC16106)	1	CLSI	>8	>8	>8
<i>Cryptococcus gattii</i> (Clinical isolate)	1	EUCAST	0.25	0.125	0.5
<i>Cryptococcus neoformans</i> (ATCC24067)	1	CLSI	0.008	0.016	0.016

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<i>Lichtheimia corymbifera</i> (ATCC7909)	1	CLSI	>8	>8	>8
<i>Mucor circinelloides</i> (ATCC8542)	1	CLSI	>8	>8	>8
<i>Rhizomucor pusillus</i> (ATCC16458)	1	CLSI	>8	>8	>8
<i>Rhizopus oryzae</i> (ATCC11145)	1	CLSI	2	>8	>8

772 <sup>a</sup> Due to the limited number of strains tested, the mean of isolate MICs is presented.

773 <sup>b</sup> MIC given is MIC<sub>50</sub>.

774 AR = azole resistant, fluconazole and voriconazole.

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791 **TABLE 7** Potency and persistence of action of PC945, posaconazole and voriconazole

Test Agent	Hyphae			BEAS2B		
	IC <sub>50</sub> (µg/ml)		Fold change	IC <sub>50</sub> (µg/ml)		Fold change
	Non-washout	Washout		Non-washout	Washout	
<b>PC945</b>	0.00010*	0.000086	x0.87	0.0037	0.043	x11.5
<b>Voriconazole</b>	0.011	>1	x >93	0.054	>1	x >18.6
<b>Posaconazole</b>	0.00045	0.0022	x4.90	0.0031	0.046	x14.7

792 \* P < 0.05; PC945 versus the results for voriconazole (Kruskal-Wallis one-way ANOVA with

793 Dunn's test).

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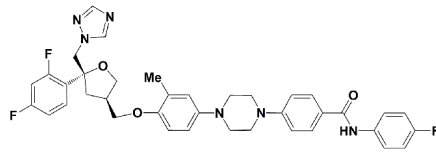
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810 **TABLE 8** *In vivo* activities of PC945 and posaconazole

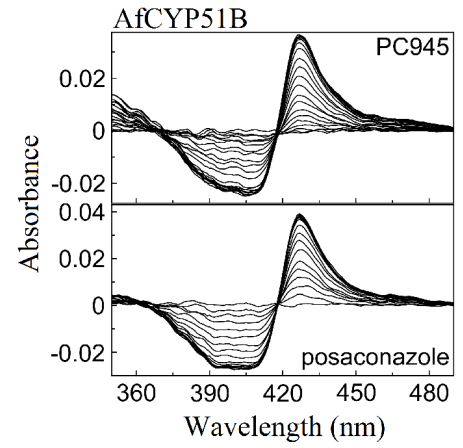
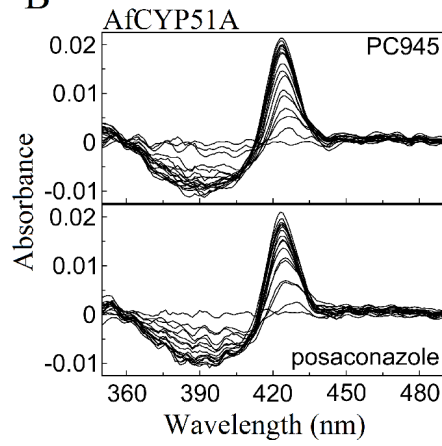
Test Agent	Dose ( $\mu\text{g}/\text{mouse}$ )	Survivor on DAY7 n/N (%)	Median survival day	Log-rank (Mantel-Cox Test) p-value
<b>Vehicle</b>	-	3/16 (19)	5	-
<b>PC945</b>	0.56	4/8 (50)	6	0.14
	2.8	5/8 (63)	6.5	0.022*
	14	10/16 (63)	undefined	0.0095**
<b>Posaconazole</b>	2.8	1/8 (13)	5	0.69
	14	7/16 (44)	6	0.050*
	70	6/8 (75)	undefined	0.0028**

811 n = number; N = total.

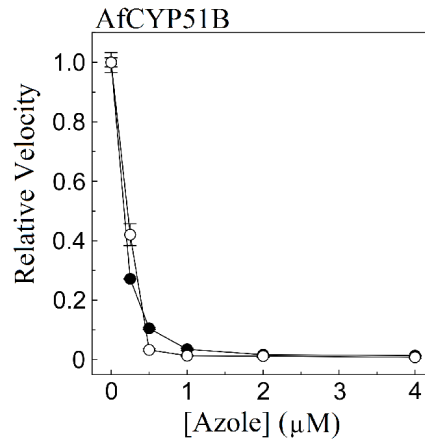
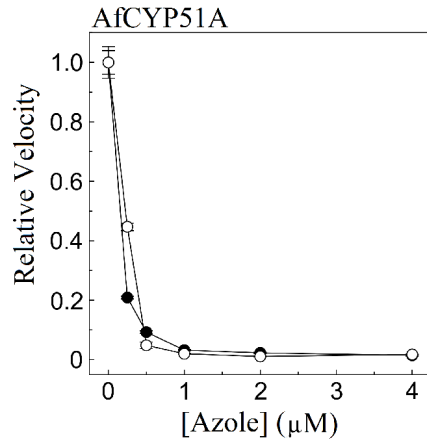
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