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Title

# *In Vitro* and *In Vivo* Efficacy of a Novel and Long Acting Fungicidal Azole, PC1244 on *Aspergillus fumigatus* Infection

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## ABSTRACT

The antifungal effects of the novel triazole, PC1244, designed for topical or inhaled administration, against *A. fumigatus* have been tested in a range of *in vitro* and *in vivo* studies. PC1244 demonstrated potent antifungal activities against clinical *A. fumigatus* isolates (N=96) with a MIC range of 0.016–0.25 μg/ml, whereas the MIC range for voriconazole was 0.25–0.5 μg/ml. PC1244 was a strong tight-binding inhibitor of recombinant *A. fumigatus* CYP51A and CYP51B (sterol 14α-demethylase) enzymes and strongly inhibited ergosterol synthesis in *A. fumigatus* with an $IC_{50}$ of 8 nM. PC1244 was effective against a broad spectrum of pathogenic fungi (MIC ranged from <0.0078~2 μg/ml), especially on *Aspergillus terreus*, *Trichophyton rubrum*, *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Cryptococcus gattii*, *Cryptococcus neoformans and Rhizopus oryzae*. PC1244 also proved to be quickly absorbed into both *A. fumigatus* hyphae and bronchial epithelial cells, producing persistent antifungal effects. In addition, PC1244 showed fungicidal activity (MFC, 2 μg/ml), which was 8-fold more potent than voriconazole. *In vivo*, once daily intranasal administration of PC1244 (3.2 ~ 80μg/mL) to temporarily neutropenic, immunocompromised mice 24h after inoculation with itraconazole-susceptible *A. fumigatus* substantially reduced fungal load in the lung, galactomannan in serum and circulating inflammatory cytokines. Furthermore, 7 days extended prophylaxis with PC1244 showed superior *in vivo* effects when compared against 1 day of prophylactic treatment, suggesting accumulation of the effects of PC1244. Thus, PC1244 has the potential to be a novel therapy for the treatment of *A. fumigatus* infection in the lungs of humans.

## INTRODUCTION

The incidence of fungal infections has increased substantially over the past two decades and invasive forms are leading causes of morbidity and mortality, especially amongst immunocompromised or immunosuppressed patients. In addition, chronic lung infections with *Aspergillus*, such as a previous infection with tuberculosis (1) or pulmonary inflammatory diseases, can leave patients with poor lung function, and extensive and permanent lung structual change (2-4).

Systemic triazole therapy is the basis for treating infections with pathogenic fungi but the adverse effects of itraconazole (ITC), voriconazole (VRC) and posaconazole (POS) are well characterised and thought to be a consequence of the pharmacological effects of the compounds in host tissues (5-9). It has been observed that up to 15% of patients treated with voriconazole experience raised transaminase levels in the liver, a site of triazole toxicity (10, 11). Serious unwanted effects in other organs have been reported after oral or systemic VRC and POS treatment, and exposure of the liver also results in significant drug interactions arising from triazole inhibition of hepatic P450 enzymes (12, 13), although recent azoles isavuconazole and VT1161 showed better risk-benefit profiles in clinical or preclinical tests (14, 15).

Administration of triazoles orally can lead to wide variations in patient response due to variable plasma concentrations, leading to compromised individual efficacy (16). Furthermore notable drug interactions for voriconazole due to the inhibition of hepatic P450 enzymes make clinical use challenging and indeed the variability in exposure of the triazoles via the oral route necessitates the need for close therapeutic drug monitoring and limits the use of triazole therapy prophylactically in at risk groups (13, 16). In addition, structural changes in the lung architecture, caused by chronic pulmonary disease or infection with tuberculosis, can lead to *Aspergillus*

colonisation of pre-existing cavities, limiting the efficacy of orally administered compounds which often struggle to penetrate into the pulmonary epithelial lining fluid (17). It is acknowledged that targeted administration to the lung, the primary point of infection, would prolong lung tissue residence and reduce systemic exposure, to display a better risk-benefit ratio. Recently, existing antifungal medications such as AMB, VRC and ITC have been repurposed in this manner to effectively prevent invasive disease (18-20).

In this report we disclose the *in vitro* and *in vivo* activities of a newly discovered azole class, antifungal agent: 4-(4-(4-(((3*R*,5*R*)-5-((1*H*-1,2,4-triazol-1-yl)methyl)-5-(2,4-difluorophenyl)tetrahydrofuran-3-yl)methoxy)-3-methylphenyl)piperazin-1-yl)-*N*-((1*S*,2*S*)-2-hydroxycyclohexyl)benzamide, (referred to here as PC1244, Fig 1) (21). The compound demonstrates activities comparable to POS and superior to VRC against both ITC susceptible and resistant strains, and it has been designed to have physicochemical properties suitable for topical administration to the lung and promote long lasting tissue residency.

## RESULTS

***In vitro* antifungal activity against laboratory adopted strains of *A. fumigatus*.** The antifungal activity of test compounds against *A. fumigatus* strains (Itraconazole (ITC) susceptible-NCPF2010, AF294, AF293; ITC-resistant-AF72, AF91) were calculated from growth curves generated by spectrophotometric analysis and compared to positive and negative controls.

It was observed that significantly lower concentrations of PC1244 were needed for endpoints (50% inhibition ($IC_{50}$ [OD]) and 90% inhibition ($IC_{90}$ [OD]) than those of all reference compounds, including voriconazole (VRC), posaconazole (POS) and itraconazole (ITC), against ITC-susceptible *A. fumigatus* laboratory strains (NCPF2010, AF294, AF293, Table 1) (22, 23). In addition, PC1244 was the most active test agent against known ITC-resistant *A. fumigatus* strains (AF72, AF91) (24, 25) (Table 1).

***In vitro* antifungal activity against clinically isolated *A. fumigatus*.** The antifungal activity of PC1244 was further evaluated in 96 clinical isolates [obtained from the Saint Louis Hospital, Paris, France (50 isolates) and NW Mycology Centre, Manchester, UK (46 isolates)]. In this study, PC1244 was found to be 6.2-fold more potent than VRC and demonstrated comparable effects to POS based on their geometric means (Table 2, Fig 2). During this assay, the quality control strain *A. fumigatus* ATCC204305 was used for validation and posaconazole showed a MIC of 0.25 µg/ml, within the range set by the EUCAST guidelines.

***In vitro* assessment of antifungal activity using CLSI methodology.** *A. fumigatus* growth inhibition by PC1244 was confirmed by the CLSI method as well as the EUCAST microdilution

method. Following the CLSI methodology guidelines (M38-A (26)), the growth of four ITC-susceptible laboratory *A. fumigatus* strains was assessed visually. PC1244 generated a MIC (0.063 μg/ml) which was comparable to POS (0.047 μg/ml) and 8-fold more potent than VRC (0.5 μg/ml).

**CYP51 binding properties.** Both PC1244 and POS produced type II difference spectra when titrated against purified recombinant *A. fumigatus* (AF293) CYP51A and CYP51B enzymes. PC1244 bound with similar affinities to the two isoenzymes as POS (CYP51A, $K_d$ values 0.74 and 0.96 μM for PC1244 and POS, respectively; CYP51B, $K_d$ values of 0.018 and 0.012 μM, respectively) (Fig 3A, B). The low-end accuracy limit for $K_d$ determinations using the modified Morrison equation is ~0.5 to 1% of the enzyme concentration (27), i.e. 0.020 to 0.040 μM in this study. Consequently, calculated $K_d$ values below 0.020 μM should be treated numerically as <0.020 μM.

**Inhibitory activity against *A. fumigatus* CYP51 enzyme.** The inhibitory activities of PC1244 against *A. fumigatus* sterol 14α-demethylases were determined using 0.5 μM *A. fumigatus* CYP51A and 0.5 μM CYP51B in the membrane fraction prepared from *Escherichia coli* expression clones, and compared to those of posaconazole. Both PC1244 and POS were strong tight-binding inhibitors of CYP51A and CYP51B *in vitro* activity (CYP51A $IC_{50}$ values for PC1244 and POS: 0.27 and 0.16 μM; and CYP51B $IC_{50}$ values of 0.23 and 0.17 μM, respectively), suggesting $K_{i.app}$ values below 1 nM (27), with PC1244 being equally as effective as POS (Fig 3C, D). This data suggests that both agents share the same mode of action; by directly coordinating as the sixth axial ligand of the CYP51 heme iron.









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**Cell based *A. fumigatus* sterol composition and CYP51 assay.** The sterol composition of *A. fumigatus* (NCPF2010) was determined for cells treated with 0, 0.0001, 0.001, 0.01, 0.1, and 1 µg/ml PC1244 and posaconazole. Sterols were extracted by saponification with KOH followed by extraction with n-hexane and TMS-derivatisation prior to analysis by GC/MS. Azole treatment of *A. fumigatus* resulted in the dose dependent accumulation of the 14 α-methylated sterols (lanosterol and eburicol) and the corresponding depletion of the final sterol product: ergosterol (Table 3), characteristic of cellular CYP51 inhibition.

CYP51 enzyme inhibitory activity was also measured in a cell-based assay, as described previously (28). In this plate-based ergosterol quantification experiment, oxidation of ergosterol by cholesterol oxidase was determined by observing the conversion of the weakly fluorescent resazurin to the highly fluorescent resorufin, and was normalised using crystal violet staining (indicating cell number). Mirroring the inhibitory activity observed in the cell-free model of CYP51 and the sterol profiles of treated cells, PC1244 strongly inhibited ergosterol production ($IC_{50}$ = 0.0055 µg/ml; 0.0080 µM) and was 12-fold more potent than VRC ($IC_{50}$ = 0.067 µg/ml; 0.19 µM) and 2.2-fold more potent than POS ($IC_{50}$ = 0.012 µg/ml; 0.017 µM).

***In vitro* determination of persistence of action.** The duration of action of test agents within the hyphae of *A. fumigatus* has been determined using a resazurin-based microtiter assay (28). *A. fumigatus* hyphae were exposed to test agents for 16 h and the inhibition of fungal growth was measured, and the efficacy was compared with that obtained after contact with drug for only 20 minutes, followed by washout and incubation for the same period. As seen in Table 4, it was observed that PC1244 ($IC_{50}$: 0.00011 µg/ml) was 100 and 4.1-fold more potent than VRC and POS, respectively, at inhibiting hyphal *A. fumigatus* growth. In addition, the potency of VRC and

157 POS diminished markedly, after short contact and washout, by factors of >93-fold and 4.9-fold,
158 respectively. In contrast, washout produced only a 2.4-fold reduction in the activity of PC1244
159 compared with continuous contact in this experimental paradigm (Table 4, Fig 4A and B).

160 In a second system, the persistence of action of the same three agents on *A. fumigatus*-
161 infected bronchial epithelial cells was quantified using GM production in the culture supernatant
162 as an index of fungal growth. BEAS2B cells were infected with *A. fumigatus*, and the effects of a
163 24 h washout period (media change) prior to infection were examined. A one hour contact time
164 with PC1244 followed by 24 h washout resulted in a 5.4-fold loss of potency against *A.*
165 *fumigatus*, compared with the control where there was no washout. POS showed a greater loss of
166 its activity on washout (14.7-fold) and it was particularly notable that VRC was ineffective under
167 the same test conditions (Table 4, Fig 4C and D). The pattern of effects mirror those seen in *A.*
168 *fumigatus* hyphae (above) and imply that only a short contact period of bronchial epithelial cells
169 with PC1244 would be required for the agent to exert a long duration of therapeutic action.
170
171 ***In vitro* fungicidal activity against *A. fumigatus*.** The MFC for each compound was calculated
172 48 h after supernatants from the MIC assay were transferred to agar plates, and determined to be
173 the lowest concentration of compound that yielded 3 colonies or less (CFU-MFC). PC1244
174 exhibited the greatest CFU-MFC of all compounds tested (2 µg/ml), and was 2 and 8-fold
175 stronger than POS and VRC, respectively. The ratios of CFU-MFC versus MIC were 32, 32 and
176 9.6 for PC1244, POS and VRC, respectively (Table 5).

177 In addition, the fungicidal effect of each compound was determined using an XTT based
178 quantitative colorimetric analysis (XTT-MFC). Absorbance was measured at $OD_{450\text{-}620}$, 24 h after
179 supernatant including compound was removed from the wells used for broth microdilution MIC
180 assay. Again, PC1244 exhibited the greatest level of inhibition of all compounds tested with a









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XTT-MFC of 0.14 µg/ml. In this system, PC1244 was 3-fold more potent than POS and >229-fold more potent than VRC. The maximum inhibition of fungicidal activities for PC1244, POS and VRC were 99.9% at 1 µg/ml, 100% at 2 µg/ml and 69.2% at 32 µg/ml, respectively. In addition, the ratios of XTT-MFC versus MIC were 2.2, 3.4 and >19 for PC1244, POS and VRC, respectively (Table 5, Fig 5).

***In vivo* antifungal activity on ITC susceptible *A. fumigatus* infection.** To assess the *in vivo* activity of PC1244, temporarily neutropenic mice infected with *A. fumigatus* (ATCC13073 [strain: NIH 5233]) were used. MIC values of PC1244, POS, VRC and ITC against this strain were 0.063, 0.125, 0.5 and 0.5 µg/ml, respectively. An aqueous suspension of PC1244 in isotonic saline (0.0032, 0.016 and 0.08 mg/ml, 35µl, please see the table 6 for conversion to mg/mouse or approximately mg/kg) was dosed by intranasal injection once daily for 3 days post infection with ITC-susceptible *A. fumigatus*. This "late intervention" regimen was found to strongly inhibit fungal load (CFU) in the lung, the highest dose (0.08 mg/ml) administered exhibiting 97% inhibition, when compared to vehicle (Fig 6A). In comparison, POS, given at the same level of 0.08 mg/ml, achieved only 39% inhibition of lung fungal load and the $ID_{Log10}$ value (the dose to reduce 1 log10 of CFU/g) was 2.0 mg/ml (70µg/mouse)) which was 143-fold higher than that of PC1244 ($ID_{Log10}$ : 0.014 mg/ml (0.49µg/mouse)). PC1244 also decreased GM concentrations in BALF in a dose-dependent manner, showing $ID_{50}$ value of 0.032 mg/ml (1.1 µg/mouse), which was 6.7fold lower than that of POS ($ID_{50}$: 0.21 mg/ml (7.4 µg/mouse)) (Fig 6B). PC1244 decreased GM concentrations in serum in a dose-dependent manner, too (Fig 4C). Notably, 0.08 mg/ml (2.8 µg/mouse) of PC1244 produced marked inhibition (82% inhibition) of GM in serum, whereas POS, at the same dose, did not show any effect (-11% inhibition). In pilot study, we also measured *A. fumigatus* PCR products in lung tissue, and consistent with these data

above, PC1244 inhibited the accumulation of PCR product(Supplement Fig 1B). In addition, PC1244 also reduced *A.fumigatus* infection dependent increase in CXCL1 in BALF (Fig 6D), IL-6 (Fig 6E) and TNFα levels (Fig 6E4D) in serum.

Extended prophylaxis with PC1244 [0.0032 mg/ml (0.11 μg/mouse); -7/0] achieved a high level of inhibitory effects on fungal load and biomarkers when compared to that observed in the late intervention study (Day 1-3 treatment) at the same dose (Fig 6A vs. 7A, Fig 6C vs. Fig 7B). Furthermore, a marked difference between extended prophylaxis (-7/0) and a shorter period of treatment (-1/0) was also observed on CFU in lung, galactomannan in serum and MDA (malondialdehyde, an oxidative stress marker) in BALF (Fig 7A, B and C).

As we indicated persistent action of PC1244 in the *in vitro* system eralier, the persistent action was also evaluated *in vivo*. As the MIC value on the *A. fumigatus* strain (ATCC13073) used in this study was 2-fold lower in PC1244 than POS, PC1244 at 0.4 mg/ml (14 μg/mouse) and POS at 0.8 mg/ml (28 μg/mouse) were intranasally administered 16 h before *A. fumigatus* inoculation, and the lungs were collected for GM and CFU assessment 8 h after *A. fumigatus* inoculation. As observed in Fig. 8A and B, PC1244 showed significant inhibition on both GM and CFU in the lung, but POS did not despite of administration at a 2-fold higher dose. Thus, in the *in vivo* system, persistent action of PC1244 was confirmed.

**Antifungal activity against non-*A. fumigatus* species.** The *in vitro* activity of PC1244 was compared with VRC, and POS against 23 pathogenic fungi (1~2 isolates each) and the results are displayed in Table 7. In all non-*Aspergillus* and non-*Candida* species tested, PC1244 was more potent or comparable in potency to POS and VRC. Of particular note, PC1244 was effective (MIC, 0.25 – 2 μg/ml) against species in which VRC and POS had no effect within the concentration range tested (MIC, >8 μg/ml), including *Gibberella zeae (Fusarium*

*graminearum)*, *Lichtheimia corymbifera*, *Mucor circinelloides*, *Rhizomucor pusillus* and *Rhizopus oryzae*. PC1244 was found to have antifungal activity against *A. flavus*, *A. niger*, and *A. terreus*, albeit with less potency than POS. Against *Aspergillus carbonarius*, PC1244 was equally potent to POS and more potent than VRC. Against all *Candida* species tested (*Candida albicans*, *Candida glabrata*, *Candida krusei* and *Candida parapsilosis*), PC1244 was more potent than VRC, and stronger or comparable in potency to POS in its inhibitory activity.

## DISCUSSION

In this report, we present data demonstrating that: 1. the novel triazole PC1244 possesses both potent and persistent antifungal activity and significant fungicidal activity against ITC susceptible and/or ITC resistant *A. fumigatus in vitro*, 2. the antifungal activity was confirmed in clinical isolates from two geographical areas, 3. intranasal once-daily PC1244 treatment exhibited potent antifungal effects against *A. fumigatus in vivo*, in temporarily neutropenic mice, 4. PC1244 showed a broad range of antifungal activity when screened against a panel of pathogenic fungal organisms.

The proposed mechanism of action of PC1244 is inhibition of sterol 14α-demethylase (CYP51A1), the enzyme required to convert eburicol to 14-demethylated eburicol, an essential step in the ergosterol biosynthesis pathway in fungi. Type II binding spectra, which display an $A_{max}$ at 423-430 nm and a broad trough at 386-412 nm arise through a specific interaction in which the triazole *N*-4 nitrogen (posaconazole) or the imadazole ring *N*-3 nitrogen coordinates as the sixth axial ligand with the heme iron to form a low-spin CYP51-azole complex (29, 30). PC1244 produced type II difference spectra when titrated against purified recombinant ITC-susceptible *A. fumigatus* (AF293) CYP51A and CYP51B, and bound with a similar affinity to

both enzymes as posaconazole. Furthermore, the strong inhibition of CYP51A activity observed with both PC1244 and posaconazole, characteristic of tight-binding inhibitors ($IC_{50}$ value approximately half that of the enzyme concentration present), exceeded that predicted by the calculated $K_d$ values from ligand binding studies using recombinant CYP51A, suggesting that the conformation of purified CYP51A in solution differs from that in cell membranes.

In sterol composition determinations, treatment with increasing concentrations of either PC1244 or posaconazole, from 0 to 1 µg/ml, resulted in an accumulation of the 14-methylated sterols, lanosterol and eburicol, and depletion of the final sterol product, ergosterol; this pattern of effect is consistent with CYP51 inhibition being the key pharmacological activity of both agents. In addition, a cell-based assay of ergosterol biosynthesis in *A. fumigatus* demonstrated that PC1244 was 12 and 2.2-fold more potent at inhibiting ergosterol production than voriconazole and posaconazole, respectively. Thus, the mechanism of action of PC1244, as for other triazole antifungals, is the inhibition of fungal sterol 14α-demethylase, resulting in the depletion of ergosterol in the fungal membrane so disrupting membrane structure and function and inhibiting growth of the pathogenic organism (31).

A highly desirable feature of topical medicines is a long duration of action ensuring that the desired therapeutic activity is maintained throughout the inter-dose period. This is particularly relevant to the treatment of pulmonary infection with *A. fumigatus*, which germinates in both extracellular environments and intracellular compartments. The duration of action of PC1244 was therefore considered an important property and was evaluated in a variety of *in vitro* systems. In *A. fumigatus* hyphae, the $IC_{50}$ value measured for PC1244 following a 20 minute contact period and washout for 16 h was reduced only 2.4-fold relative to that obtained following continuous contact with the drug for the same period without washout. Furthermore, in the BEAS2B cell line, washout for 24 h, after a 1 h contact period, resulted in only an approximate

276 5-fold loss of potency against *A. fumigatus* compared with control. These observed properties of
277 rapid cellular penetration and persistence of action, in the absence of the pathogen, may be
278 particularly valuable characteristics, which enhance the potential use of PC1244 in prophylaxis.
279 The persistent action of PC1244 was also confirmed in the *in vivo* system when administered 16
280 h before *A. fumigatus* inoculation (Figure 5).

281 In the *in vivo* system, intranasal treatment of PC1244 showed better effects than
282 posaconazole despite comparable MIC values in *in vitro* testing. We speculate that, firstly,
283 persistence of the drug substance on bronchial cells, as shown in Figure 4, is likely to be a
284 contributory factor to the amplification of the antifungal effects of PC1244 *in vivo* seen in the
285 current once daily treatment regimen. Secondly, we have demonstrated here that a 7 day
286 extended prophylactic treatment (using very low doses) produced much greater anti-*Aspergillus*
287 activity than prophylactic treatment for 1 day, and also that the effects of 7 day prophylactic
288 treatment are maintained if treatment ceased when *Aspergillus* is inoculated on day 0 (Fig. 7).
289 This is powerful pharmacodynamic evidence that the effects of PC1244 accumulate on daily
290 dosing in mice and are maintained when dosing is terminated. Thirdly, Baistrocchi and
291 colleagues published evidence of the accumulation of posaconazole in granulocyte type cells and
292 demonstrated enhanced synergic antifungal effects (by exposure of *Aspergillus* to cellular
293 posaconazole during phagocytosis) (32). Considering the persistent action of PC1244, it is likely
294 that granulocytes/macrophages containing PC1244 contributed to further enhancement of the
295 antifungal effect.

296 Determining whether an antifungal compound is “fungistatic” or “fungicidal” is complex
297 and the clinical utility of such characterisation is the subject of much debate. Fungal infections of
298 body compartments that are not easily accessed by host defences require agents that are
299 fungicidal in nature, and this is especially true in immunocompromised patients (33). For an

antibiotic to qualify as bactericidal its minimum bactericidal concentration (MBC) must be no more than 2× to 4× the MIC, but the definition of "fungicidal" is yet to be standardised (33). MFC determination in filamentous fungi is not standardised either, but studies have shown that reproducible MFCs can be obtained by following standardised broth microdilution methods for MIC determination, followed by subculture onto agar (33, 34). Defining an MFC as the lowest drug dilution to yield less than 3 colonies to obtain 99% - 99.5% killing activity, Espinel-Ingroff *et al* determined the $MFC_{90}$ range of itraconazole (0.2 – 4 μg/ml), voriconazole (0.5 – 4 μg/ml) and posaconazole (0.06 – 2 μg/ml), in a number of *A. fumigatus* isolates (34). However, it is worth noting that the agar subculture methodology tests for fungicidal activity on planktonic Af growth only. Here we have used a combination of different methodologies to attempt to determine the fungicidal activity of PC1244 and clinically used triazoles accurately. Using subculture on agar (CFU-MFC) gave an MFC of 2 μg/ml for PC1244 with an MFC/MIC ratio of 32, and similar results were seen with posaconazole (MFC = 4 μg/ml, MFC/MIC = 32), whilst voriconazole exhibited a higher MFC (16 μg/ml) but a lower MFC/MIC ratio (9.6). As discussed above, this data would suggest voriconazole is a more fungicidal compound as it exhibits a lower MFC/MIC ratio. However, recent studies have shown that this technique likely overestimates the fungicidal activity of a compound as it does not factor in viable conidia attached to the base of test wells (35). To account for this phenomenon, a colorimetric method for assessing the fungicidal activity of a compound against sessile *A. fumigatus* was used (35). MFC determination by this microbroth colorimetric method (XTT-MFC) gave an MFC of 0.14 μg/ml for PC1244 with an MFC/MIC ratio of 2.2, which was superior to both posaconazole (MFC = 0.42 μg/ml, MFC/MIC = 3.4) and voriconazole (MFC = >32 μg/ml, MFC/MIC = >19). Therefore, with these data we provide evidence that PC1244 is a fungicidal compound with a similar or improved degree of potency to posaconazole.

As with any study there are limitations, especially the *in vivo* study. Firstly, the delivery system does not mimic clinical use. The advantage of intranasal instillation is being able to confirm that all the solution is delivered into the body, but we do not control the level of lung exposure or the exposure site (same as aerosolization). However, we carefully optimised the intranasal dosing volume, as it has been shown that approximately 60% of the administered dose will be deposited in the lung after intranasal treatment (36) and also we confirmed trachea/lung deposition after intranasal administration of 35 µL of methylene blue solution to A/J mice (data not shown). Aerosolization with close drug monitoring at the exposure site (rather than systemic) is ideal, but this is not easily achieved as special imaging equipment is required, as Miller *et al* demonstrated elegantly using whole-Animal Luminescent Imaging (37). Secondly, CFUs were determined in only whole left lobe. This will cause location bias of fungal load, and ideally we should test in homogenate from the whole lung. However, when we determined CFU and GM in right lobe and left lobe, we did not find any significant difference of these biomarkers between right lobe and left lobe (data not shown). In addition, to avoid this bias, we determined GM in BALF and serum. Thirdly, there was a lack of pharmacokinetic measurement of PC1244 in mice used for the *in vivo* study. PC1244 has been optimised for topical treatment to the lung to maximize local exposure and minimise systemic exposure. Systemic concentrations of drug are therefore not a useful surrogate marker to help explain the different antifungal efficacy of compounds. However, we have some data demonstrating that measurable levels of systemic exposure do occur. In preliminary studies using non-infected mice dosed intra-tracheally with 40 µL of a 2 mg/ml aqueous suspension of PC1244, it was shown that the plasma concentrations of PC1244 ranged from 111 ~ 303 ng/ml 2 hours post dose, and 249 ~ 339 ng/ml 8 hours post dose. But this had been reduced to 41.5 ~ 50.7 ng/ml 24 hours post dose despite decent *in vivo* effects after once daily treatment. Under the same conditions, the plasma concentrations achieved with

348 posaconazole were 15.6 ~ 125 ng/ml at 24 hours post dose, which was more variable but not too
349 different from that of PC1244, although the *in vivo* activity of PC1244 was superior to that of
350 posaconazole. As PC1244 has much less oral availability compared with posaconazole
351 (unpublished data), the exposure results from absorption through the respiratory tract (not by
352 accidental ingestion of compound during dosing). Furthermore, all compounds are water
353 insoluble, and administered topically (exposed to respiratory tract directly). We do not, therefore,
354 believe that water solubility is a key factor explaining the *in vivo* efficacies or topical exposure
355 levels. Finally, we tested only limited isolates of azole resistant strains. Further study will be
356 required with a wide range of clinical azole resistant isolates with different genotypes to
357 determine its potency against recent *A. fumigatus* strains with $TR_{34}$/L98H and
358 $TR_{46}$/Y121F/T289A mutations or other genetic cause(s) underlying resistance.
359 Thus, due to its superior or comparable activity against both azole susceptible and azole
360 resistant *A. fumigatus*, persistent action, extended retention within the lung after topical treatment
361 and broad repertoire of fungal targets, PC1244 has the potential to become a valuable new
362 therapeutic agent for the treatment of *A. fumigatus* and other, difficult to treat, fungal infections
363 in man.

## MATERIALS & METHODS

**Antifungal agents.** PC1244 was synthesised by Sygnature Discovery Ltd (Nottingham, UK), and voriconazole (Tokyo Chemical Industry UK Ltd., Oxford, UK), posaconazole (Apichem Chemical Technology Co., Ltd., Zhejiang, China), itraconazole (Arkopharma, Carros, France), amphotericin B (Selleckchem, Munich, Germany) and caspofungin (Selleckchem, Munich, Germany) were procured from commercial sources. For *in vitro* antifungal assays, stock solutions of test agents were prepared in DMSO (2000 μg/ml). For *in vivo* studies, solid materials of test agents were directly suspended in physiological saline at 10 mg/ml, and diluted with physiological saline after sonication.

***A. fumigatus* CYP51 binding assay and enzyme inhibitory activity.** *A. fumigatus* CYP51 binding properties were determined as previously reported (28, 38). Test agents were titrated against 4 μM recombinant *A. fumigatus* (AF293 strain) CYP51A or CYP51B proteins and binding saturation curves were constructed from the change in the absorbance between the spectral peak and the trough. A rearrangement of the Morrison equation was used to determine the dissociation constant ($K_d$) values when ligand binding was tight (39).

A CYP51 reconstitution assay system was used to determine 50% inhibitory ($IC_{50}$) concentrations (40). Test agent was added to a mixture of 0.5 μM CYP51, 1 μM *A. fumigatus* cytochrome P450 reductase isoenzyme 1 (AfCPR1), 50 μM eburicol, 4% ($^{w}/_{v}$) 2-hydroxypropyl-β-cyclodextrin, 0.4 mg/ml isocitrate dehydrogenase, 25 mM trisodium isocitrate, 50 mM NaCl, 5 mM $MgCl_2$ and 40 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS; pH ~7.2). The mixtures were then incubated at 37°C for 10 minutes prior to initiation with 4 mM β-$NADPHNa_4$ followed by shaking for 20 minutes at 37°C. Sterol metabolites were recovered by extraction

with ethyl acetate followed by derivatisation with 0.1 ml *N*,*O*-bis(trimethylsilyl)trifluoroacetamide : trimethylchlorosilane (99:1) and 0.3 ml anhydrous pyridine prior to analysis by gas chromatography mass spectrometry.

***A. fumigatus* sterol analysis.** A working suspension of *A. fumigatus* spores (NCPF2010) was prepared in filter-sterilised MOPS RPMI-1640 (RPMI-1640 containing 2 mM L-glutamine, 2% glucose, 0.165 M MOPS, buffered to pH 7 with NaOH) at a final concentration of 8 x $10^6$ spores $ml^{-1}$. To each 100 mm Petri dish, 10 ml of the working suspension was added and the dishes were incubated for 4 h at 35°C and 5% $CO_2$. Samples for baseline determinations were collected by scraping, pelleted by centrifugation at 2000 rpm for 5 minutes and stored at -80°C. Test compounds or DMSO (50 µl) were added to the remaining dishes, which were subsequently gently rocked by hand to disperse the compounds. Dishes were incubated for 2 h at 35°C and 5% $CO_2$. Samples were collected and processed as described above. Posaconazole and PC1244 concentrations of 0.0001, 0.001, 0.01, 0.1 and 1 µg $ml^{-1}$ were tested. These samples were prepared in the laboratory at Pulmocide Ltd., and sent to the laboratory in the Centre for Cytochrome P450 Biodiversity, Institute of Life Science, Swansea University Medical School, for analysis.

Non-saponifiable lipids were extracted as previously reported (31) and were derivatised with 0.1 ml *N*,*O*-bis(trimethylsilyl)trifluoroacetamide : trimethylchlorosilane (99:1) and 0.3 ml anhydrous pyridine (2 h at 80°C) prior to analysis by gas chromatography mass spectrometry (41). Sterol composition was calculated using peak areas from the gas chromatograms and the mass fragmentation patterns compared to known standards were used to confirm sterol identity. The sterol content of *A. fumigatus* (basal) and treated *A. fumigatus* (either DMSO, posaconazole or PC1244) were determined in three biological replicates.

411

412 ***A. fumigatus* cell based ergosterol assay.** Growth medium (RPMI-1640, 2 mM L-glutamine,
413 2% glucose, 0.165 M MOPS, 0.5% BSA, pH 7.0) was added across a 96-well plate and test
414 agents were added in duplicate. *A. fumigatus* (NCPF2010) conidia were added across the plate at
415 a final concentration of 1 x $10^4$ $ml^{-1}$. After incubation for 24 h at 35°C, media was removed from
416 all wells and replaced with reaction buffer (Amplex red cholesterol assay kit, ThermoFisher,
417 A12216) and Amplex red solution. Plates were incubated for 30 minutes at 37°C, protected from
418 light, after which fluorescence was quantified using a spectrophotometer. Media was removed
419 from all wells and replaced with crystal violet solution (1% $^{v}/_{v}$), and plates were incubated at
420 room temperature on a shaker for 30 minutes. Plates were washed three times with PBS, and
421 sodium dodecyl sulphate solution (0.1% $^{v}/_{v}$) was added across the plate to lyse the cells. After
422 incubation at room temperature for 1 h, absorbance was measured at $OD_{590}$ using a
423 spectrophotometer.

424

425 ***In vitro* antifungal activity against *A. fumigatus*.** Assessment of antifungal activity against a
426 selection of *A. fumigatus* laboratory/clinical strains (NCPF2010 [National Collection of
427 Pathogenic Fungi (NCPF), Bristol, UK], AF72 [NCPF, Bristol, UK], AF91 [NCPF, Bristol, UK],
428 AF293 [NCPF, Bristol, UK], AF294 [NCPF, Bristol, UK]) was performed using EUCAST
429 methodology as previously reported (28), in a 384-well plate format as quadricates, in three
430 independent experiments. Growth medium (RPMI-1640, 2 mM L-glutamine, 2% glucose, 0.165
431 M MOPS, 0.5% BSA, pH 7.0) was added across the plate, test agents were added in
432 quadruplicate and the DMSO concentration was identical across the plates. Conidia were added
433 across the plate at a final concentration of 1 x $10^5$ $ml^{-1}$. Plates were incubated for 48 h at 35°C
434 after which turbidity was assessed by measuring optical density (OD) at 530 nm using a

spectrophotometer, and the $IC_{50}$ and $IC_{90}$ values were calculated from the concentration-response curve generated for each test compound using a four-parameter logistic equation (Dotmatics, Bishops Stortford, UK). *A. fumigatus* ATCC204305 was used as the assay control. Determination of antifungal activity against 50 *A. fumigatus* clinical isolates from St Louis Hospital (Paris, France) was performed with 96-well plates using the EUCAST method shown above (28) in duplicate. Antifungal susceptibility testing for 46 *A. fumigatus* isolates [obtained from the North West England Mycology Reference Centre] was performed as singlicate by Evotec (UK) Ltd (Manchester, UK) according to EUCAST guidelines. Assessment of the antifungal activity of four of the *A. fumigatus* strains (ATCC1028, ATCC10894, ATCC13073, and ATCC16424) was performed as singlicate according to M38-A methodology described by the Clinical and Laboratory Standards Institute (CLSI) (26) by Eurofins Panlabs Taiwan Ltd. (Taipei, Taiwan).

***In vitro* antifungal activity against other fungal species.** For the measurement of activity against *C. gattii*, the method described in EUCAST definitive document EDef 7.2 was used and assay plates were incubated statically at 37°C in ambient air for 24 h (± 2 h) unless poor growth necessitated further incubation to 36 or 48 h (42). Antifungal potency against *Aspergillus flavus*, *Aspergillus niger* and *A. terreus*, was determined as set out in EUCAST definitive document EDef 9.2 and assay plates were incubated at 37°C for 48 h (43). These tests were conducted at Evotec (UK) Ltd (Manchester, UK). Measurement of activity against other fungi was performed by Eurofins Scientific according to methodology described by the Clinical and Laboratory Standards Institute (CLSI) (CLSI M38-A (26) or M27-A2 (44), www.eurofinspanlabs.com). The source or strain name of each fungus species was indicated in Table 6. The MIC against *C.*

458 *albicans, C.parapsilosis and C.glabrata* were determined using an azole endpoint, which
459 indicates 50% inhibition of fungus growth.
460
461 ***In vitro* fungicidal activity of PC1244 against *A. fumigatus*.** The antifungal activity of PC1244
462 against *A. fumigatus* [NCPF2010] was determined in 96-well plates using the methodology
463 described above as duplicates, in three independent experiments. After the MIC for each
464 compound was recorded, fungicidal activity was determined as previously described (35).
465 Briefly, media from each well (100 µl/well) was removed after pipetting up and down five times
466 and sub-cultured onto 4% Sabouraud dextrose agar plates. The plates were incubated (35°C with
467 ambient air) for 48 h and the colony forming units (CFU) were counted for each compound
468 concentration. The minimum fungicidal concentration (MFC) was determined as the lowest
469 compound concentration yielding 3 colonies or less.
470 After removal of media for culture-based CFU testing, the contents of all wells were
471 carefully aspirated and warm PBS (200 µl/well) was added. After gentle agitation, the contents of
472 all wells were aspirated and fresh medium added (200 µl/well). The plates were incubated (35°C
473 with ambient air) for 24 h. A working solution of 0.5 mg/ml XTT and 125 µM menadione was
474 prepared in PBS and added across the plate (50 µl/well). The plates were incubated (35°C with
475 ambient air) for 2 h, after which plates were agitated gently for 2 min. The optical density (OD)
476 of each well at 450 nm and 620 nm was measured using a multi-scanner (Clariostar: BMG,
477 Buckinghamshire, UK). The MFC (XTT-MFC) was calculated from the concentration-response
478 curve generated using a cut-off of 99% inhibition.
479
480 ***In vitro* determination of persistence of action on *A. fumigatus* hyphae.** The persistence of
481 action of test agents was determined in *A. fumigatus* hyphae (NCPF2010) as previously reported

(28). Briefly, conidia diluted in growth media (RPMI-1640, 2 mM L-glutamine, 2% glucose, 0.165 M MOPS, pH 7.0) were added across a 384-well plate at a final concentration of $1x10^3$/well. After incubation at 35°C for exactly 6 h, test and reference articles or neat DMSO (as vehicle) (0.5 µl/well) were added to the appropriate wells to give a final concentration of 0.5% DMSO. The plates were incubated for exactly 20 minutes at 35°C and 5% $CO_2$. After the incubation time had elapsed all wells on the designated washout plate were aspirated and growth media (100 µl/well) was added across the plate. For the non-washout plate, after compounds were added to hyphae, no media change was applied. Resazurin (0.04% diluted in growth media) was added to all wells of both non-washout and washout plates (5 µl/well) to give a final concentration of 0.002% resazurin. The plates were incubated at 35°C and 5% $CO_2$ for 16 h. Subsequently fluorescence in each well was measured at $\lambda_{ex}/\lambda_{em}$ 545/600 nm using a multiscanner (Clariostar: BMG, Buckinghamshire, UK). The percentage inhibition for each well was calculated and the $IC_{50}$ value was calculated from the concentration-response curve generated for each test compound using a four-parameter logistic equation (Dotmatics, Bishops Stortford, UK). This study was conducted in quadricates, in three independent experiments.

***In vitro* determination of persistence of action on bronchial epithelial cells.** The persistence of action of test agents was evaluated in immortalised, bronchial, epithelial cells (BEAS2B) as previously reported (28). Each experiment consisted of one non-washout plate (96-well) and a parallel washout plate into which BEAS2Bs were seeded at a concentration of $3x10^4$ cells/well in growth media (RPMI-1640, 2 mM L-glutamine, 10% FCS), and incubated for 24 h at 37°C, 5% $CO_2$. Test and reference articles or neat DMSO (as vehicle) (0.5 µl/well) were added to the appropriate wells of the washout plate to give a final concentration of 0.5% DMSO. The plate was incubated for exactly 1 h at 37°C and 5% $CO_2$. After the incubation time had elapsed all

wells on the washout plate were aspirated and growth media (100 µl/well) was added across the plate. After 24 h incubation at 37°C, test and reference articles or neat DMSO (as vehicle) (0.5 µl/well) were added to the appropriate wells of the non-washout plate to give a final concentration of 0.5% DMSO. The plate was incubated for exactly 1 h at 37°C and 5% $CO_2$ after which *A. fumigatus* conidia were added across both plates at a final concentration of 1 x $10^3$/well. Fungal growth was determined after a further 24 h incubation at 35°C, 5% $CO_2$, by measuring galactomannan (GM) concentrations, using Platelia GM-EIA kits (Bio-Rad Laboratories, 62794). The percentage inhibition for each well was calculated and the $IC_{50}$ value was calculated from the concentration-response curve generated for each test compound using a four-parameter logistic equation (Dotmatics, Bishops Stortford, UK). This study was conducted in triplicates, in three independent experiments.

***In vivo* antifungal activity against *A. fumigatus* infection.** As previously reported (28), we tested antifungal effects of test articles on *A. fumigatus* infected, temporarily neutropenic mice. Specific pathogen-free A/J mice (male, 5 weeks old) were used for *A. fumigatus* infection as they have been described to be more susceptible to *A. fumigatus* infection previously (45). Animals (N=6 per group) were then dosed with hydrocortisone (Sigma H4881, 125 mg/kg, subcutaneously) on days 3, 2 and 1 before infection, and with cyclophosphamide (Sigma C0768; 250 mg/kg, intraperitoneally) two days before infection to induce temporary neutropenia. Both hydrocortisone and cyclophosphamide were diluted with physiological saline. To avoid bacterial infection during immunosuppression, drinking water was supplemented with tetracycline hydrochloride (Sigma T7660; 1 µg/ml) and ciprofloxacin (Fluka 17850; 64 µg/ml). Conidia of *A. fumigatus* (ATCC13073 [strain: NIH 5233]) were aseptically dislodged from the malt agar plates

and suspended in sterile distilled water with 0.05% Tween 80 and 0.1% agar. On the day of infection, 30 µl (15 µl in each nostril) of the conidia suspension ($1.67 \times 10^8$/ml in physiological saline) was administered intranasally under 3% isoflurane.

Test agents, suspended in physiological saline, were administered intranasally (35 µl, approximately 17.5 µl each in each nostril), once daily, on days 1, 2 and 3 post infection. To investigate extended prophylaxis, PC1244 was administered intranasally once daily, on days -7 to 0 and the effects were compared with treatment on days -1 to 0. As the injection volume was fixed and body weight was changed every day, especially after infection, the accurate dose unit was µg/mouse. However, as the average body weight after immunosuppression and just before infection was 20 g, we also calculated estimated dose as mg/kg. Therefore, 35 µl injections of 0.0032, 0.016, 0.08, 0.4, 2 mg/ml were equivalent to 0.11, 0.56, 2.8, 14, 70 µg/mouse, respectively, which were approximately 0.0056, 0.028, 0.14, 0.7, 3.5 mg/kg, respectively (Table 6).

A body weight loss of > 20%, compared with an animal's weight on day 1, or a mouse death, were both defined as "drop-out" events. Animals that lost > 20% of their initial body weight were sacrificed. Animals were terminally anaesthetised 6 h after the last dose of drug was administered on day 3. The volume inserted intranasally is reported to achieve almost 60% deposition into the lung (36).

BALF was collected through cannulated tracheas using physiological saline (46), blood was then collected via cardiac puncture, and lung tissue was removed for homogenate preparation. The *Aspergillus* GM concentration in serum was determined with Platelia GM-EIA kits (Bio-Rad Laboratories, 62794). The value was provided as a "cut-off index" (COI) which was calculated by the formula: COI = OD in sample / OD in cut-off control, provided by the kit. For tissue fungal load, 100 mg of whole left lobe of lung tissue was removed aseptically and homogenized







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in 0.2 ml of 0.1% agar in sterile distilled water as previously reported (28). We confirmed that the CFU level was not significantly different between the right lung and left lung. Serially diluted lung homogenates were plated on malt agar plates (50 µl/plate), and incubated at 24 ± 1°C for 72 to 96 h. The colonies of *A. fumigatus* on each plate were counted and the fungal titre presented here as CFUs ($x10^3$) per gram of lung tissue.

Measurement of TNFα and IL-6 in serum and CXCL1 in BALF was performed using Quantikine® mouse ELISA kit (R&D systems, Inc., Minneapolis, MN, USA). MDA (malondialdehyde) analysis was also performed using OxiSelect® TBARS Assay Kit (MDA Quantitation; Cell Biolabs Inc, San Diego, CA, USA). For quantitative PCR, DNA amplification was performed with Premix Ex Taq™ (Takara Bio, Kusatsu, Japan) and analysed in 96-well optical reaction plates, using the standard curve method. *A. fumigatus* 18S rRNA gene fragments were amplified with the primer pair; 5'-GGCCCTTAAATAGCCCGGT-3' and 5'-TGAGCCGATAGTCCCCCTAA-3', and hybridization probe; 5'-FAM-AGCCAGCGGCCCGCAAATG-TAMRA-3'. Each 25 µl reaction solution contained 50 ng of DNA from mice lungs and 200 nM of probe. The PCR protocol was as follows: incubation at 50°C for 2 min and 95°C for 10 min; followed by 55 cycles of 65°C for 1 min and 95°C for 15 sec. The fluorescence was monitored at the end of each cycle to obtain a measure of the amount of PCR product formed. The cycle numbers at which each sample reached the threshold were determined and the amounts of *A. fumigatus* DNA in 50 ng of mice lung DNA was evaluated from the standard curve with the cycle numbers and $\log_2$ concentrations of 0.05-50,000 pg of DNA from *A. fumigatus*. All animal studies were approved by the Ethics Review Committee for Animal Experimentation of Nihon University. *A. fumigatus* studies were approved by the Microbial Safety Management Committee of Nihon University School of Pharmacy (E-H25-001).

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578 **Statistical analysis.** Results are expressed as means ± standard error of the mean (SEM). For
579 comparison between groups either the ordinary one-way ANOVA with Tukey's *post hoc*
580 comparison or the Kruskal-Wallis ANOVA with Dunn's *post hoc* comparison test were
581 performed. Statistical significance was defined as $P<0.05$.

582

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## FIGURE LEGENDS

**FIG 1** Structure of PC1244

**FIG 2** Inhibitory activity of PC1244 against 96 clinical isolates of *A. fumigatus* in France and UK. Each horizontal bar was presented as Geometric mean with 95% confidence interval.

**FIG 3** Efficacy of PC1244 on sterol 14α-demethylase (CYP51) activity. (A, B) Type II azole binding spectra for *A. fumigatus* CYP51A (A) and CYP51B (B), each experiment was performed 4-6 times although only one replicate is shown, (C, D) Azole $IC_{50}$ determinations of posaconazole (●) and PC1244 (○), mean relative velocity values are shown with standard deviations for *A. fumigatus* CYP51A (C) and CYP51B (D).

**FIG 4** Duration of action of PC1244 against *A. fumigatus*. (A, B) Persistence of action of PC1244 (A) and voriconazole (B) on *A. fumigatus* hyphae, mean values and SEM of 3 independent experiments (each experiment was conducted in quadricate), (C, D) Persistence of action of PC1244 (C) and voriconazole (D) on human bronchial cell lines (BEAS2B) infected with *A. fumigatus*, mean values and SEM of 3 independent experiments (each experiment was conducted in triplicate).

**FIG 5** Colorimetric microbroth assessment of fungicidal activity of PC1244 against *A. fumigatus* [NCPF2010] *in vitro*. Mean values and SEM of 3 independent experiments.

**FIG 6** Antifungal activity of PC1244 against *A. fumigatus in vivo*. PC1244 (0.0032, 0.0016 and 0.08 mg/ml aqueous suspension) and posaconazole (0.08, 0.4 and 2 mg/ml aqueous suspension)

762 were intranasally given on days 1, 2 and 3 post infection of *A. fumigatus* in temporary
763 neutropenic immunocompromised mice, Fungal load (CFU/g lung tissue) in lung (A),
764 galactomannan (GM) in BALF (B) galactomannan (GM) in serum (C), CXCL1 in BALF (D),
765 IL-6 in serum (E) and TNFα in serum (F) were evaluated on day 3 post infection (N=5~6).
766 (N=6). Each horizontal bar was presented as mean ± SD from 5~6 mice per group. * $P<0.05$,
767 **P<0.01, ***p<0.001 vs. infected control. "**+**" dead before sample collection. Serum could not
768 collected from dead mice.
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770 **FIG 7** Antifungal activity of extended prophylaxis treatment of PC1244 against *A. fumigatus in*
771 *vivo*. Effects of 7 days extended prophylaxis with intranasal PC1244 was compared with that of 1
772 day prophylaxis treatment only on lung fungal load (CFU/g tissue) (A), GM (COI) in serum (B)
773 and malondialdehyde (MDA) in BALF (C) of *A. fumigatus* infected immunocompromised mice
774 (N=4~5). Each horizontal bar was presented as mean ± SD from 4~6 mice per group. * $P<0.05$.
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776 **FIG 8** Single prophylactic treatment of PC1244 and posaconazole against *A. fumigatus in vivo*.
777 PC1244 at 0.4 mg/ml (14 µg/mouse) and posaconazole at 0.8 mg/ml (28 µg/mouse) were
778 intranasally administered 16 h before *A. fumigatus* inoculation, and the lungs were collected for
779 galactomannan (GM, COI) (A) and fungal load (CFU/g tissue) (B) assessment at 8 h after *A.*
780 *fumigatus* inoculation. Each bar was presented as mean ± SD from 3~4 mice per group. * $P<0.05$.
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798 **TABLE 1** Antifungal effects of PC1244 and known antifungal agents in azole susceptible and azole resistant strains of *A. fumigatus* [a].

| | $IC_{50}$ ($IC_{90}$) (µg/ml) of indicated agent | | | | | |
|---|---|---|---|---|---|---|
| **Strain** | **PC1244** | **Voriconazole** | **Posaconazole** | **Itraconazole** | **Amphotericin B** | **Caspofungin** |
| NCPF2010 | 0.0017 (0.0022) | 0.15 (0.21) | 0.0070 (0.0084) | 0.037 (0.054) | 0.20 (0.62) | 0.065 (>1) |
| AF294 | 0.0021 (0.0041) | 0.083 (0.27) | 0.0056 (0.011) | 0.041 (0.052) | 0.21 (0.79) | >1 (>1) |
| AF293 | 0.0026 (0.012) | 0.25 (0.74) | 0.010 (0.028) | 0.032 (0.23) | 0.24 (0.85) | >1 (>1) |
| AF72 | 0.0024 (0.026) | 0.025 (0.066) | 0.042 (0.30) | 0.31 (>1) | 0.12 (0.42) | 0.065 (>1) |
| AF91 | 0.0037 (0.024) | 0.14 (0.28) | 0.038 (0.049) | 0.22 (>1) | 0.28 (0.75) | 0.11 (>1) |

799 [a] $IC_{50}$ and $IC_{90}$ values were determined from optical density measurements.

800 All compounds have been tested in a range of concentrations (0.002 ~ 1 µg/ml). The data are from 3 independent experiments and

801 each test was performed in quadricate.

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Antimicrobial Agents and Chemotherapy

809 **TABLE 2** *In vitro* activities of PC1244, posaconazole and voriconazole against 96 clinically
810 isolated *A. fumigatus* strains [a].

| | MIC (µg/ml)[b] | | | | |
|---|---|---|---|---|---|
| **Test Agent** | **Range** | **Geometric mean** | **Mode** | **$MIC_{50}$** | **$MIC_{90}$** |
| **PC1244** | 0.008 – 2 | 0.067**** | 0.016 | 0.032 | 0.50 |
| **Voriconazole** | 0.06 – 4 | 0.42 | 0.50 | 0.50 | 1.0 |
| **Posaconazole** | 0.016 – 2 | 0.10**** | 0.032 | 0.063 | 0.50 |

811 [a]All MIC were determined visually; $MIC_{50}$ and $MIC_{90}$ values represent the concentrations
812 required to inhibit 50 and 90% of the strains tested.

813 [b]****, $P < 0.0001$; versus the results for voriconazole (One way ANOVA with Tukey's test).

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829 **TABLE 3** Mean percentage sterol composition of *A. fumigatus* treated with either posaconazole

830 (A) or PC1244 (B) of three biological replicates (± standard deviation from the mean).

| **(A) Sterol** | **Sterol compositions (posaconazole-treated [µg/ml])** | | | | | |
|---|---|---|---|---|---|---|
| | **DMSO** | **0.0001** | **0.001** | **0.01** | **0.1** | **1** |
| **Ergosterol** | 100 (± 0) | 94.5 (±0.1) | 87.2 (±0.7) | 74.7 (±0.8) | 67.8 (±0.3) | 67.4 (±1.5) |
| **Ergost-5,7-dienol** | 0 | 3.3 (±1.9) | 3.9 (±0.6) | 0 | 0 | 0 |
| **Lanosterol** | 0 | 0 | 3.0 (±0.9) | 7.0 (±0.3) | 8.8 (±0.5) | 8.8 (±0.4) |
| **Eburicol** | 0 | 2.2 (±1.2) | 5.9 (±0.5) | 18.3 (±1.1) | 23.4 (±0.8) | 23.8 (±1.3) |

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| **(B) Sterol** | **Sterol compositions (PC1244-treated [µg/ml])** | | | | | |
|---|---|---|---|---|---|---|
| | **DMSO** | **0.0001** | **0.001** | **0.01** | **0.1** | **1** |
| **Ergosterol** | 100 (± 0) | 91.3 (±0.1) | 89.2 (±0.2) | 76.8 (±2.6) | 61.0 (±1.6) | 58.7 (±2.2) |
| **Ergost-5,7-dienol** | 0 | 4.6 (±1.8) | 4.6(±0.8) | 0 | 0 | 0 |
| **Lanosterol** | 0 | 1.7 (±0.7) | 2.8 (±0.6) | 8.5 (±1.1) | 12.3 (±0.8) | 13.1 (±0.9) |
| **Eburicol** | 0 | 2.5 (±1.1) | 3.4 (±1.2) | 14.7(±1.5) | 26.7 (±0.8) | 28.2 (±1.3) |

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845 **TABLE 4** Potencies and persistence of action of PC1244, posaconazole and voriconazole in *A.*
846 *fumigatus* hyphae and in BEAS2B cells infected with *A. fumigatus*.

| | **Hyphae** | | | **BEAS2B** | | |
|---|---|---|---|---|---|---|
| | **$IC_{50}$ (µg/ml)[a]** | | | **$IC_{50}$ (µg/ml)** | | |
| **Test Agent** | **No washout** | **Washout** | **Fold change** | **No washout** | **Washout** | **Fold change** |
| **PC1244** | 0.00011* | 0.00025 | 2.41 | 0.0034* | 0.018 | 5.40 |
| **Voriconazole** | 0.011 | >1 | >93 | 0.054 | >1 | >18.6 |
| **Posaconazole** | 0.00045 | 0.0022 | 4.90 | 0.0031 | 0.046 | 14.7 |

847 [a]*, $P < 0.05$ for PC1244 versus the results for voriconazole (Kruskal-Wallis one-way ANOVA
848 with Dunn's test).
849 Data are from 3 independent experiments, and each assay was conducted in quadricate for
850 hyphae assay and in triplicate for BEAS2B assay.
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863 **TABLE 5** Mean fungicidal activity of PC1244, posaconazole and voriconazole against *A.*

864 *fumigatus* (NCPF2010) of three biological replicates (± standard deviation from the mean)..

| | MIC/MFC (μg/ml)[b] [MFC/MIC ratio] | | |
|---|---|---|---|
| **Test Agent** | **MIC** | **CFU-MFC** | **XTT-MFC** |
| **PC1244** | 0.063 ± 0 | 2 [32] | 0.14 [2.2] |
| **Voriconazole** | 1.67 ± 0.58 | 16 [9.6] | >32 [>19] |
| **Posaconazole** | 0.125 ± 0 | 4 [32] | 0.42 [3.4] |

865 Data are from 3 independent experiments, and each assay was conducted in duplicate.

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870 **TABLE 6** Conversion of units of dose give to mice *in vivo* study

| mg/ml aqueous suspension | mg/mouse | approx.mg/kg[1] |
|---|---|---|
| 0.0032 | 0.00011 | 0.0056 |
| 0.016 | 0.00056 | 0.028 |
| 0.08 | 0.0028 | 0.14 |
| 0.4 | 0.014 | 0.70 |
| 0.8 | 0.028 | 1.4 |
| 2 | 0.07 | 3.5 |

871 1. 20g is used for calculation as average body weight of used mice

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888 **TABLE 7** Antifungal effects of PC1244, voriconazole and posaconazole on other fungal species.

| Species (Strain[s]) | Strains tested | Culture method | MIC (μg/ml) [a] | | |
|---|---|---|---|---|---|
| | | | PC1244 | Voriconazole | Posaconazole |
| *Aspergillus carbonarius* (ATCC8740) [d] | 1 | CLSI | 0.063 | 0.5 | 0.063 |
| *Aspergillus flavus* (ATCC204304) [d] | 1 | CLSI | 0.25 | 2 | 0.13 |
| *Aspergillus flavus* (AFL8; NRRC3357) | 2 | EUCAST | 0.38 | 0.63 | 0.16 |
| *Aspergillus niger* (ATCC1015) | 1 | EUCAST | 0.5 | 1 | 0.20 |
| *Aspergillus terreus* (AT49; AT7130) | 2 | EUCAST | 0.38 | 1 | 0.093 |
| *Penicillium chrysogenum* (ATCC9480) [d] | 1 | CLSI | 0.13 | 2 | 0.13 |
| *Penicillium citrinum* (ATCC9849) [d] | 1 | CLSI | 0.5 | >8 | 0.5 |
| *Trichophyton rubrum* (ATCC10218) [d] | 1 | CLSI | 0.031 | 0.063 | 0.031 |
| *Aureobasidium pullulans* (ATCC9348) [d] | 1 | CLSI | 1 | >8 | 1 |
| *Cladosporium argillaceum* (ATCC38013) [d] | 1 | CLSI | 0.25 | 0.5 | 0.25 |
| *Candida albicans* (20240.047; ATCC 10231) [d] | 2 | CLSI | <0.0078 [b] | 0.14 [b] | 0.081 [b] |
| *Candida albicans-AR* [c] (20183.073; 20186.025) [d] | 2 | CLSI | (0.25, <0.0078) [b] | 10 [b] | 8.13 [b] |
| *Candida glabrata* (ATCC 36583; R363) [d] | 2 | CLSI | (<0.0078, 0.13) [b] | 8.13 [b] | 0.5 [b] |
| *Candida krusei* (ATCC6258) [d] | 1 | CLSI | 0.13 | 0.25 | 0.125 |
| *Candida parapsilosis* (ATCC22019) [d] | 1 | CLSI | 0.25 [b] | NT | 0.25 [b] |
| *Chaetomium globosum* (ATCC44699) [d] | 1 | CLSI | 0.063 | 1 | 0.25 |
| *Gibberella zeae (Fusarium graminearum)* (ATCC16106) [d] | 1 | CLSI | 1 | >8 | >8 |
| *Cryptococcus gattii* (Clinical isolate) | 1 | EUCAST | 0.5 | 0.125 | 0.5 |

| | | | | | |
|---|---|---|---|---|---|
| *Cryptococcus neoformans* (ATCC24067) [d] | 1 | CLSI | 0.016 | 0.016 | 0.016 |
| *Lichtheimia corymbifera* (ATCC 7909) [d] | 1 | CLSI | 1 | >8 | >8 |
| *Mucor circinelloides* (ATCC8542) [d] | 1 | CLSI | 2 | >8 | >8 |
| *Rhizomucor pusillus* (ATCC16458) [d] | 1 | CLSI | 2 | >8 | >8 |
| *Rhizopus oryzae* (ATCC11145) [d] | 1 | CLSI | 0.25 | >8 | >8 |

889 [a] Due to the limited number of strains tested, the mean of isolate MICs is presented.

890 [b] MIC indicates 50% inhibition of fungal growth as azole readout.

891 [c]AR, azole resistant (fluconazole and voriconazole).

892 [d] All details of isolate and assay protocol are described in https://www.eurofinspanlabs.com

893 (Anti-infective assay/Fungi)

894 NT = not tested.

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Antimicrobial Agents and Chemotherapy

**FIGURE 1**

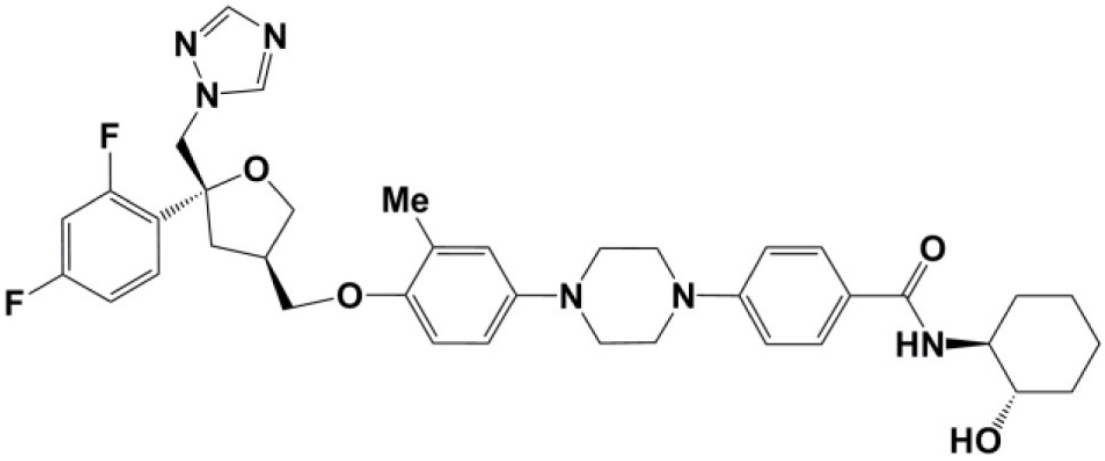


**FIGURE 2**

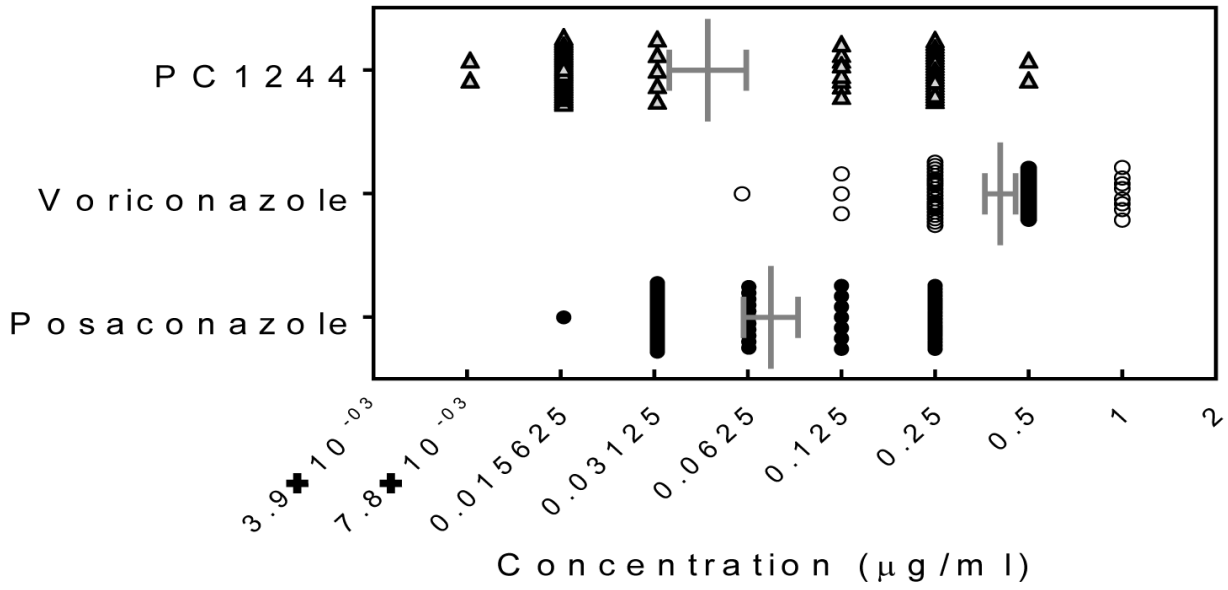


**FIGURE 3**

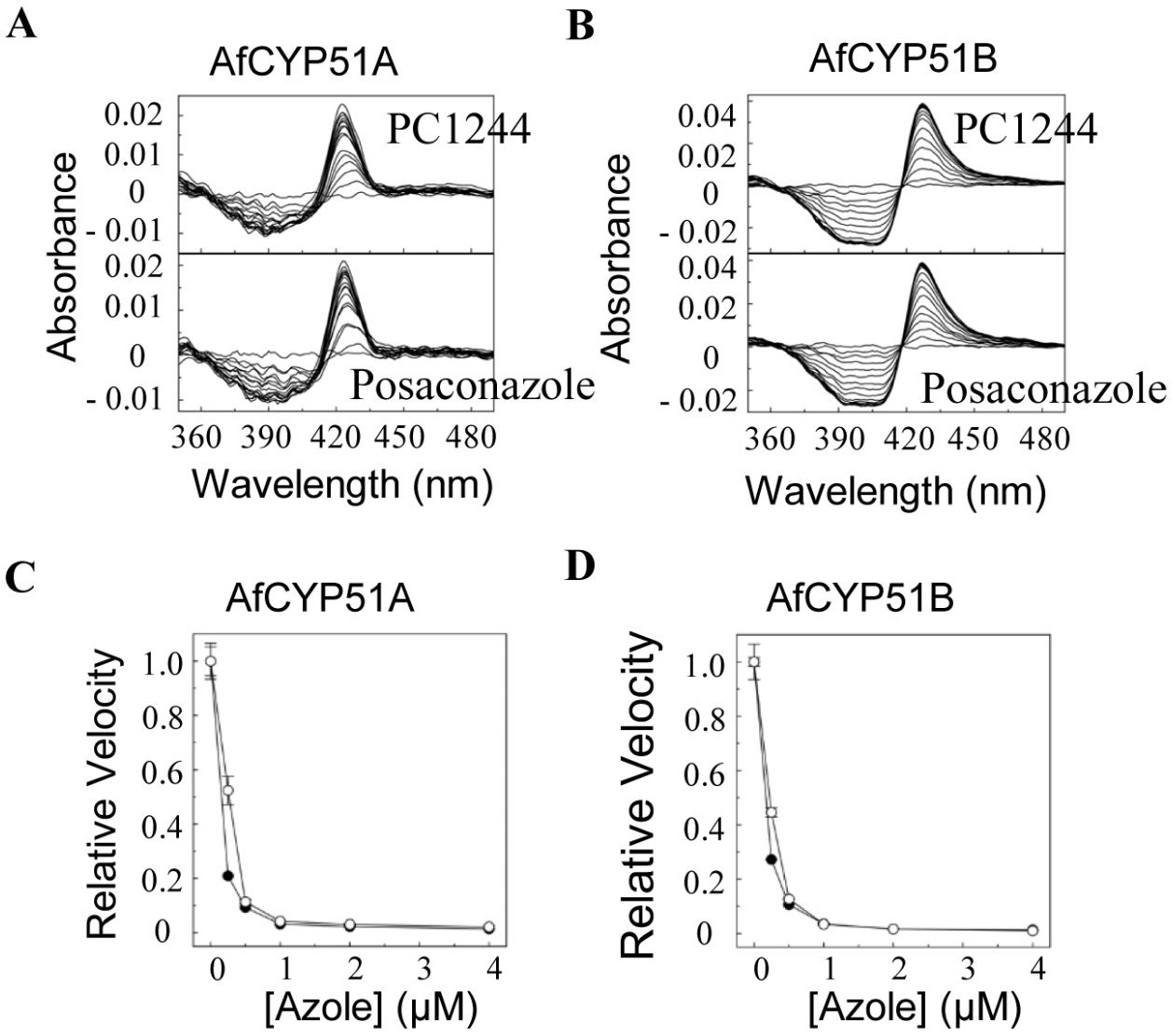
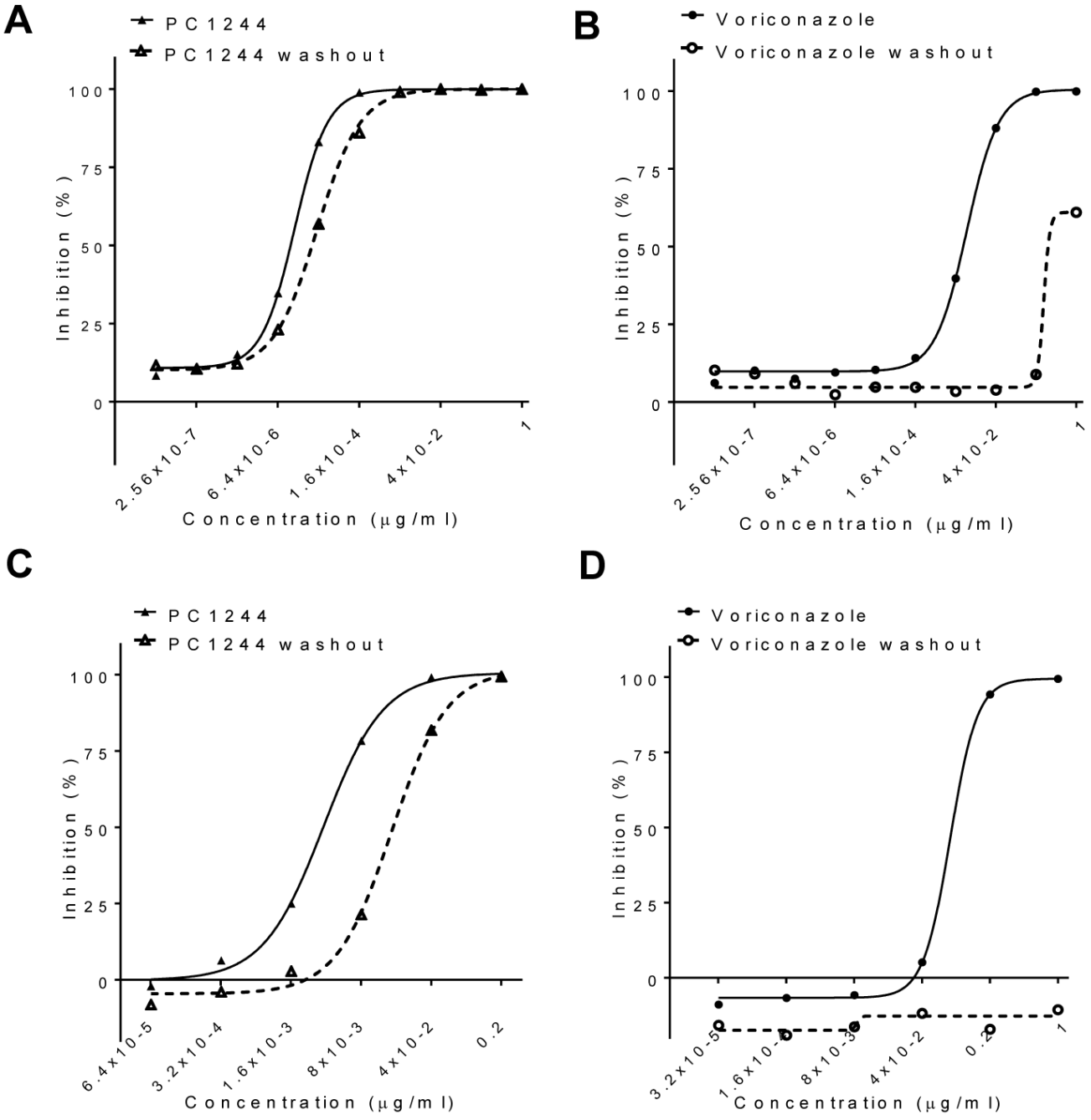


**FIGURE 4**



# FIGURE 5

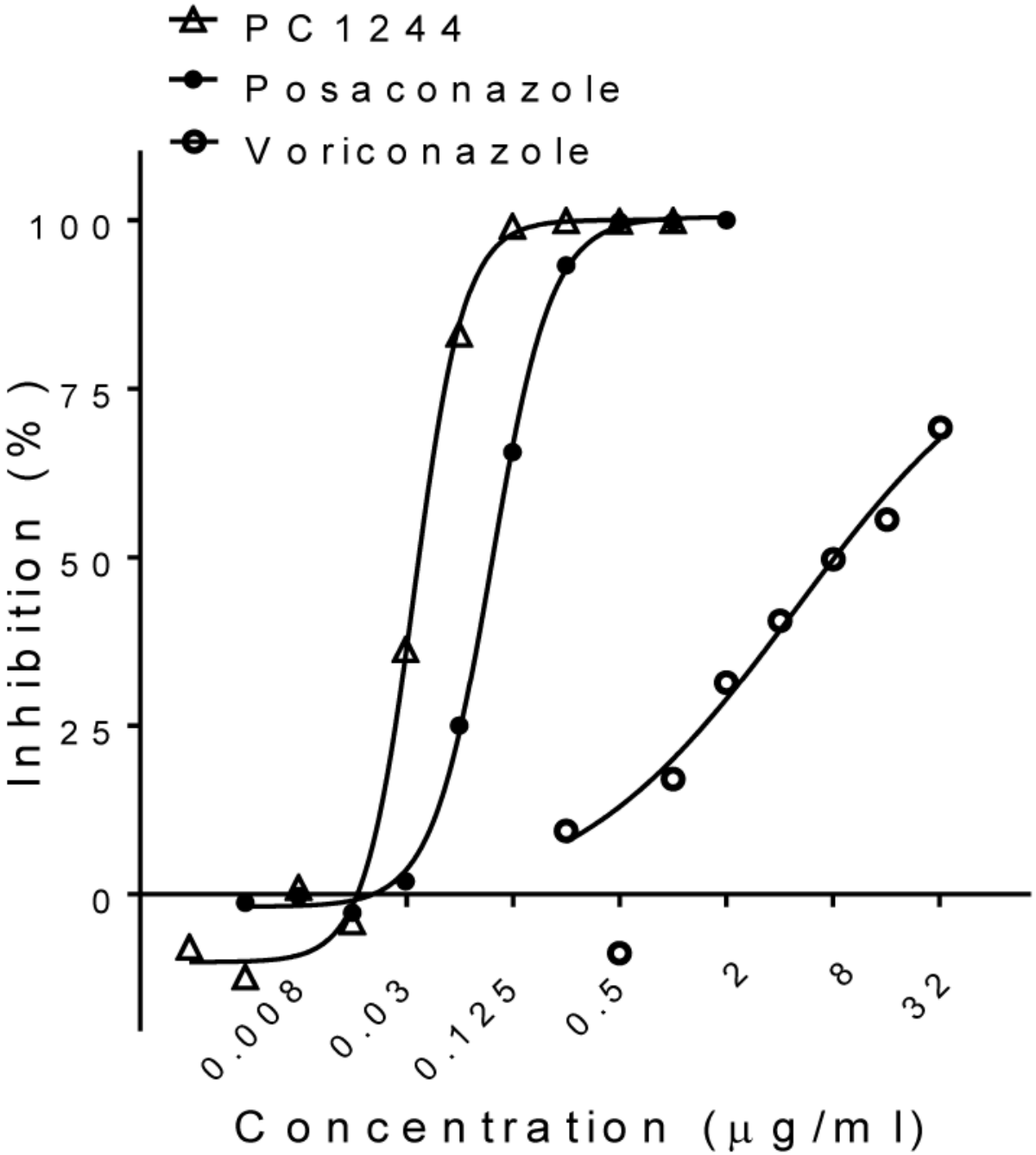


**FIGURE 6**

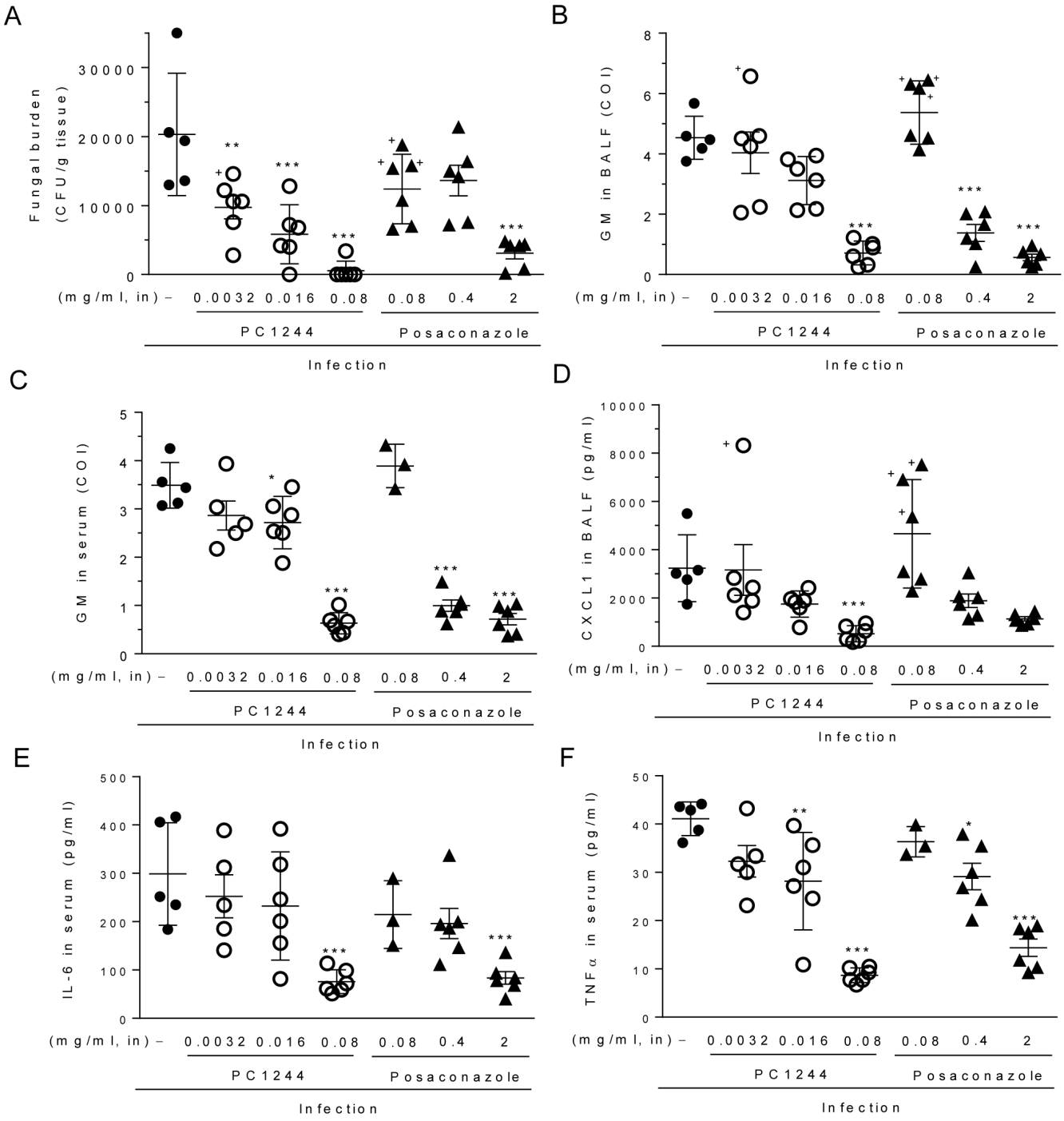


**FIGURE 7**

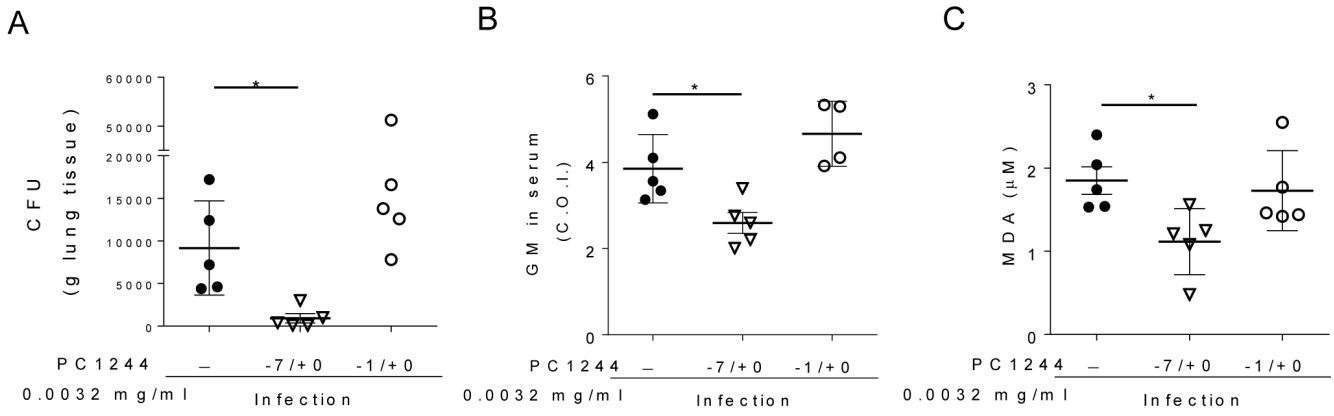


**FIGURE 8**