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Anti-fungal activity of a novel triazole, PC1244, against emerging azole-resistant *Aspergillus fumigatus* and other species of *Aspergillus*

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Objectives: The growing emergence of azole-resistant *Aspergillus fumigatus* strains worldwide is a major concern for current systemic antifungal treatment. Here we report antifungal activities of a novel inhaled triazole, PC1244, against a collection of multi-azole-resistant *A. fumigatus* strains.

Methods: MICs of PC1244 were determined for *A. fumigatus* carrying TR_{34}/L98H (n=81), TR_{46}/Y121F/T289A (n=24), M220 (n=6), G54 (n=11), TR_{53} (n=1), $TR_{46}{}^{3}$/Y121F/T289A (n=2), G448S (n=1), G432C (n=1) and P216S (n=1) resistance alleles originating from either India, the Netherlands or France. The effects of PC1244 were confirmed in an *in vitro* model of the human alveolus and *in vivo* in temporarily neutropenic, immunocompromised mice.

Results: PC1244 exhibited potent inhibition [geometric mean MIC (range), 1.0 mg/L (0.125 to >8 mg/L)] of growth of *A. fumigatus* strains carrying *cyp51A* gene mutations, showing much greater potency than voriconazole [15 mg/L (0.5 to >16 mg/L)], and an effect similar to those on other azole-susceptible *Aspergillus* spp. (*Aspergillus flavus, Aspergillus terreus, Aspergillus tubingensis, Aspergillus nidulans, Aspergillus niger, Aspergillus nomius, Aspergillus tamarii*) (0.18–1 mg/L). In TR_{34}/L98H and TR_{46}/Y121F/T289A *A. fumigatus*-infected *in vitro* human alveolus models, PC1244 achieved superior inhibition (IC_{50}, 0.25 and 0.34 mg/L, respectively) compared with that of voriconazole (IC_{90}, >3 mg/L and >10 mg/L, respectively). *In vivo*, once-daily intranasal administration of PC1244 (0.56–70 μg/mouse) to the *A. fumigatus* (AF91 with M220V)-infected mice reduced pulmonary fungal load and serum galactomannan more than intranasal posaconazole.

Conclusions: PC1244 has the potential to become a novel topical treatment of azole-resistant pulmonary aspergillosis.

Introduction

The widespread use of azole antifungal agents, both in the clinic and in agriculture, has led to a growing and problematic emergence of resistant mycoses.[1–3] Azole resistance is not limited to one particular *Aspergillus* disease entity but can occur in all of the three major forms of aspergillosis: invasive aspergillosis (IA), chronic pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis (ABPA).[4] *Aspergillus fumigatus* isolates with environmentally acquired resistance mechanisms, including TR_{34}/L98H and TR_{46}/Y121F/T289A, were first discovered in the Netherlands in 1998 but are now recognized to be a worldwide

problem.[5] Furthermore, isolates with G54 and M220 point mutations in the *cyp51A* gene, first identified as acquired by patients over a course of azole treatment, have recently been discovered in the environment.[6,7]

PC1244, which is 4-(4-(4-(((3R,5R)-5-((1H-1,2,4-triazol-1-yl)methyl)-5-(2,4-difluorophenyl)tetrahydrofuran-3-yl)methoxy)-3-methylphenyl)piperazin-1-yl)-*N*-((1S,2S)-2-hydroxycyclohexyl) benzamide, is a novel triazole antifungal agent specifically designed for inhaled administration, which has demonstrated potent, broad and persistent antifungal activities *in vitro* and *in vivo*.[8] We evaluated the antifungal activity of PC1244 against a large collection of itraconazole- or pan-azole-resistant strains of clinical *A. fumigatus* carrying TR_{34}/L98H, TR_{46}/Y121F/T289A, M220, G54, TR_{53}, $TR_{46}{}^{3}$/Y121F/T289A, G448S, G432C and P216S resistance alleles originating from India, the Netherlands and France.

Materials and methods

Antifungal agents

PC1244 was synthesized by Sygnature Discovery Ltd (Nottingham, UK) and voriconazole by 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 at 2000 mg/L. 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.

In vitro susceptibility testing of A. fumigatus

The EUCAST method was used for assays performed in the UK and France and the CLSI method for those performed in India. In each case, the method used was the laboratory's own standard.

The first assessment of antifungal activity against a selection of *A. fumigatus* laboratory/clinical strains {NCPF2010 [National Collection of Pathogenic Fungi (NCPF), Salisbury, UK]; AF72 [NCPF]; AF91 [NCPF]; AF293 [NCPF]; AF294 [NCPF]; a strain carrying TR_{34}/L98H [Saint Louis Hospital, Paris, France[9]]; and a strain carrying TR_{46}/Y121F/T289A [Vallabhbhai Patel Chest Institute, Delhi, India]} was performed by quantitative spectrophotometer growth assessment using a 384-well plate-formatted EUCAST method as previously reported.[10] Culture medium (RPMI-1640, 2 mM L-glutamine, 2% glucose, 0.165 M MOPS, 0.5% BSA, pH 7.0) was added across the plate, test agents were added in quadruplicate and the DMSO concentration was identical across the plates (0.5% DMSO in final). Conidia were added across the plate at a final concentration of 1×10^5/mL. Plates were incubated for 48 h at 35°C, after which turbidity was assessed by measuring 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* ATCC 204305 was used as the assay control.

We also determined MIC values for clinical isolates of *A. fumigatus* carrying WT *cyp51A* (n=17), TR_{34}/L98H (n=73), TR_{46}/Y121F/T289A (n=24), M220 (n=6), G54 (n=11), TR_{53} (n=1), $TR_{46}{}^{3}$/Y121F/T289A (n=2), G448S (n=1), G432C (n=1) or P216S (n=1) resistance alleles, originating from India and the Netherlands,[11] using the CLSI M38-A2 method[12] by visual reading, and also against *A. fumigatus* carrying TR_{34}/L98H (n=8) isolated in France using the 96-well-formatted EUCAST E.Def 9.3 method.[8,13] ATCC 204305 and AF293 *A. fumigatus* strains were used for quality control.

Antifungal activity against A. fumigatus infection in an in vitro model of the human alveolus

The effect of PC1244 on inhibition of *A. fumigatus* penetration through a bilayer of human cells was evaluated using an *in vitro* model of the human alveolus. Construction of the cell bilayer was performed as described previously.[14,15] Briefly, human pulmonary artery endothelial cells (HPAEC) (Lonza, Basel, Switzerland) were diluted to 1×10^6 cells/mL in EGM-2 [endothelial basal medium (EBM) supplemented with ascorbic acid, heparin, hydrocortisone, human endothelial growth factor (EGF), 2% FBS, vascular EGF, human fibroblast growth factor-B, R3-insulin-like growth factor-1, and gentamicin; Lonza, Basel, Switzerland]. Transwells (#3415; Sigma–Aldrich, Dorset, UK) were inverted and cell suspension (100 μL/well) was applied to the base of each transwell. The inverted transwells were incubated at room temperature within a flow hood for 2 h, after which they were turned upright. EGM-2 was added to the lower (600 μL/well) and upper (100 μL/well) compartments and the transwells were incubated for 48 h (37°C, 5% CO_2). The EGM-2 medium in the lower chamber was then replaced with fresh EGM-2. A549 cells were diluted to 5×10^5 cells/mL in EBM supplemented with 10% FBS and added to the upper chamber (100 μL/well) of all transwells and the plates were incubated for 72 h (37°C, 5% CO_2). On the day of inoculation, transwells were pre-treated with test and reference agents for 1 h prior to infection with itraconazole-susceptible (NCPF2010) or multi-azole-resistant (TR_{34}/L98H or TR_{46}/Y121F/T289A) *A. fumigatus* conidia (1×10^5 spores/mL; 10 μL/well). Fungal invasion into the lower chamber was determined after 24 h incubation at 35°C, 5% CO_2, by measuring galactomannan (GM) concentrations, using Platelia GM-EIA kits (lot 62794, Bio-Rad Laboratories). The percentage inhibition for each well was calculated using following formula: % inhibition=100×{1- [GM (OD) in treated group]/[GM (OD) in control infection group]}; GM was measured in the bottom chamber as the marker of invasion.

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).

In vivo antifungal activity against azole-resistant A. fumigatus infection (M220V)

As previously reported,[10] we tested antifungal effects of compounds 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.[8,16] Animals were then injected 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) 2 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, lot 17850; 64 μg/mL). Conidia of *A. fumigatus* [NCPF7100 (AF91)] 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 (1.67×10^8/mL in physiological saline) was administered intranasally under 3% isoflurane.

As PC1244 and posaconazole have limited water solubility (Table S1, available as Supplementary data at *JAC* Online), both were suspended in physiological saline and administered intranasally (35 μL in total, ~17.5 μL each in each nostril) as an aqueous suspension, once daily, on days 1, 2 and 3 post-infection. As the injection volume was fixed and body weight 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 0.016, 0.08, 0.4 and 2.0 mg/mL were

Table 1. Antifungal effects of PC1244 and known antifungal agents in azole-resistant strains of *A. fumigatus*

		IC_{50} (IC_{90}) values[a] for agent indicated (mg/L)					
Strain	Resistance mechanism	PC1244	voriconazole	posaconazole	itraconazole	amphotericin B	caspofungin
NCPF2010	none	0.0017 (0.0022)	0.15 (0.21)	0.0070 (0.0084)	0.037 (0.054)	0.20 (0.62)	0.065 (>1)
L98H (F)	TR_{34}/L98H	0.014 (0.024)	0.83 (>1)	0.050 (0.096)	0.29 (>1)	0.097 (0.39)	0.13 (>1)
TR46 (H)	TR_{46}/Y121F/T289A	0.021 (0.17)	>1 (>1)	0.098 (0.63)	0.24 (0.71)	0.077 (0.34)	0.40 (>1)
TR46 (V)	TR_{46}/Y121F/T289A	0.042 (0.12)	>1 (>1)	0.13 (0.35)	0.25 (0.55)	0.10 (0.33)	0.12 (>1)

F, France (clinical); H, Himachal Pradesh, India (environmental); V, Varanasi, India (environmental).
MIC analysis has been conducted by quantitative spectrophotometer growth assessment using a 384-well plate-formatted EUCAST method.
[a]IC_{50} and IC_{90} values were determined from OD measurements.

equivalent to 0.56, 2.8, 14 and 70 μg/mouse, respectively, which were approximately 0.028, 0.14, 0.70 and 3.5 mg/kg, respectively.

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 anaesthetized 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.[17]

Bronchoalveolar lavage fluid (BALF) was collected through cannulated tracheas using physiological saline;[18] 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, lot 62794, Radmont, WA, USA). 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 in 0.2 mL of 0.1% agar in sterile distilled water as previously reported.[8] 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 cfu ($\times 10^3$) per gram of lung tissue. 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).

Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). For comparison between groups (unpaired) either the ordinary one-way ANOVA with Tukey's *post hoc* comparison, the Kruskal–Wallis ANOVA with Dunn's *post hoc* comparison or the Wilcoxon–Mann–Whitney test was performed. For comparison of MICs between groups (paired) for the broth microdilution test, the non-parametric Friedman test with Dunn's *post hoc* multiple comparison was performed. Statistical significance was defined as $P<0.05$.

Results

Comparison of in vitro antifungal activity of PC1244 against azole-resistant A. fumigatus with registered antifungal drugs

Firstly, we evaluated antifungal activity of PC1244 against representative azole-resistant *A. fumigatus* strains, along with that of conventional antifungal agents, using quantitative spectrophotometer growth assessment. PC1244 was found to be the most potent agent, with 50% growth inhibition [IC_{50} (OD)] and 90% inhibition [IC_{90} (OD)] evaluated against itraconazole/voriconazole-resistant (TR_{34}/L98H) and itraconazole/voriconazole/posaconazole-resistant (TR_{46}/Y121F/T289A) *A. fumigatus* strains (Table 1, Figure 1a–c).Voriconazole was ineffective against either TR_{34}/L98H or TR_{46}/Y121F/T289A strains up to 1 mg/L. PC1244 showed potent inhibitory activity (IC_{90}: 0.024–0.17 mg/L), being 4- to >42-fold more potent than itraconazole and 1.7- to 4-fold more potent than posaconazole.

In a standard CLSI assay, the activity of PC1244 against *A. fumigatus* isolates with WT *cyp51A* (geometric mean MIC: 0.21 mg/L) was found to be significantly superior to voriconazole (geometric mean MIC: 0.48 mg/L) and comparable to that of posaconazole (geometric mean MIC: 0.18 mg/L) (Table 2). For comparison, the antifungal activity of PC1244 was evaluated against clinically isolated *A. fumigatus* carrying the TR_{34}/L98H (n=73), TR_{46}/Y121F/T289A (n=24), M220 (n=6), G54 (n=11), TR_{53} (n=1), TR_{46}[3]/Y121F/T289A (n=2), G448S (n=1), G432C (n=1) or P216S (n=1) resistance alleles originating from either India, the Netherlands or France. PC1244 showed different susceptibility to different mutants (Table 2) but, overall (in all mutations), PC1244 exhibited much greater potency [geometric mean MIC (range), 1.0 mg/L (0.125 to >8)] than voriconazole [15 mg/L (0.5 to >16)] and comparable to posaconazole [0.94 mg/L (0.125 to >8)]. The antifungal activity of PC1244 against *cyp51A* mutants was also found to be only 4.8-fold weaker than the activity against isolates with WT *cyp51A* (n=17), whereas a 15-fold reduction of antifungal activity was observed for voriconazole.

For TR_{34}/L98H strains, two isolates had high MICs (>8 mg/L) for posaconazole and also two isolates showed high MICs for PC1244, but those strains were not cross-resistant to posaconazole or PC1244, suggesting PC1244 and posaconazole can be subject to different mechanisms of resistance (Figure 1d). In addition, in eight TR_{34}/L98H strains obtained from France, the performance of PC1244 [geometric mean MIC (range), 0.16 mg/L (0.125–0.5)] was confirmed to be superior to voriconazole and comparable to posaconazole when assayed using the EUCAST method (Table 2), as observed in strains from the Netherlands and India.

TR_{46}/Y121F/T289A strains were also completely resistant to voriconazole in the CLSI assay (Table 2, Figure 1c). Although posaconazole was ineffective against one strain, PC1244 inhibited the growth of all isolates consistently (Figure 1e). For M220, PC1244 showed a slightly lower GM-MIC versus posaconazole. However,

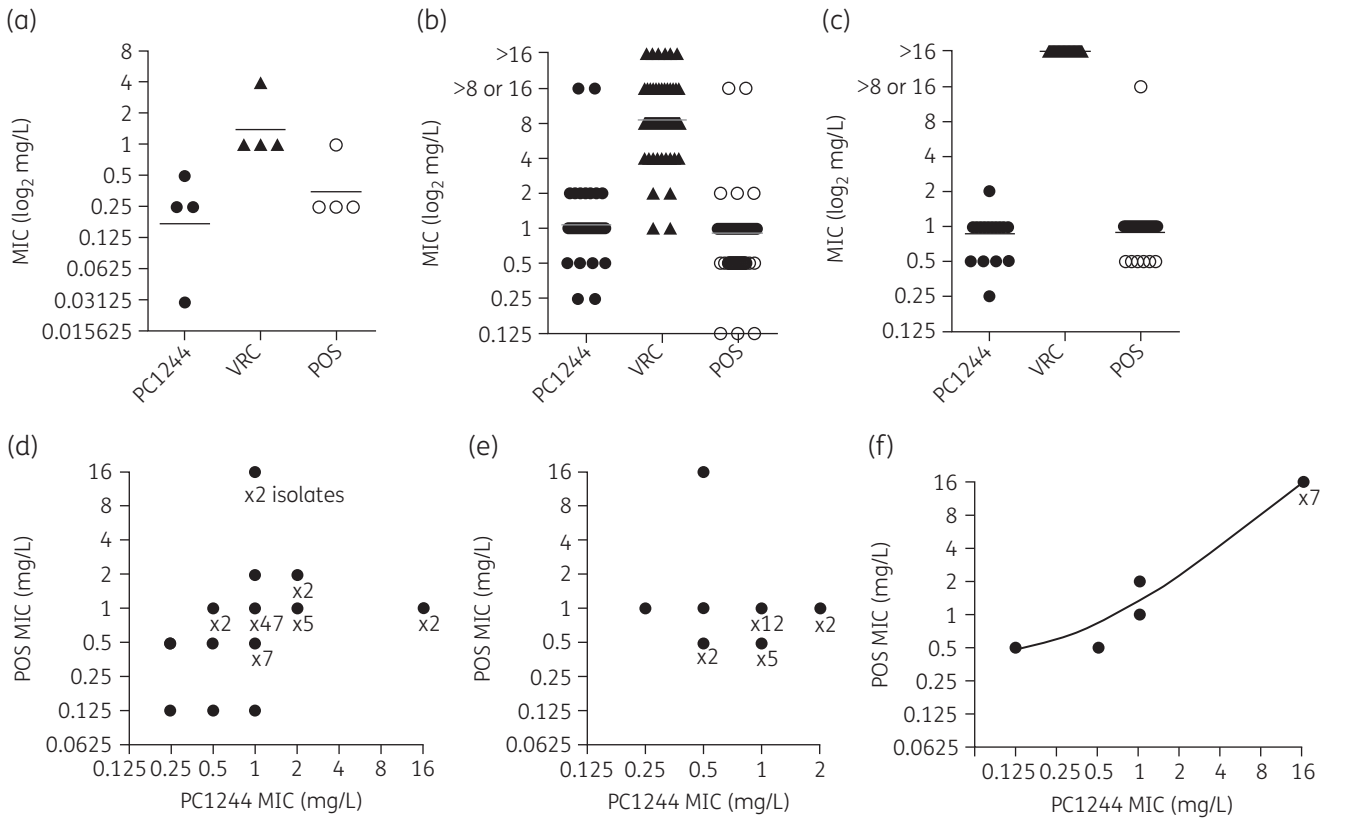

Figure 1. MIC distribution of PC1244, voriconazole (VRC) and posaconazole (POS) against azole-susceptible and -resistant *A. fumigatus* strains (CLSI). Distribution of MIC of PC1244 (closed circles), voriconazole (closed triangles) and posaconazole (open circles) against WT (a), TR_{34}/L98H (b) and TR_{46}/Y121F/T289A (c). Relationship between MICs of PC1244 and POS against TR_{34}/L98H (d), TR_{46}/Y121F/T289A (e) and G54 (f). A closed circle in panels d, e and f represents the potencies of the two compounds versus a particular *Aspergillus* strain.

against G54 isolates voriconazole outperformed both PC1244 and posaconazole, and there was good correlation between the effects of PC1244 and those of posaconazole (Figure 1f).

During this assay, the quality control strain *A. fumigatus* ATCC 204305 was used for validation, yielding an MIC of 0.25 mg/L for posaconazole and 1 mg/L for voriconazole.

Comparison of in vitro antifungal activity of PC1244 against other Aspergillus species

We also evaluated the antifungal activity of PC1244 against *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus tubingensis*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus nomius* and *Aspergillus tamarii* using the CLSI method. PC1244 showed potent activity against these azole-susceptible *Aspergillus* spp. with geometric mean MIC of 0.18–1 mg/L (Table 3), comparable to posaconazole (geometric mean MIC: 0.13–0.37 mg/L) and voriconazole (geometric mean MIC: 0.29–0.76 mg/L).

Antifungal activity against Aspergillus in an in vitro infection model of the human alveolus

An *in vitro* model of human alveoli, consisting of a bilayer of human alveolar epithelial and endothelial cells, was used to investigate the antifungal activity of PC1244 versus penetration of the bilayer by either azole-susceptible *A. fumigatus* (NCPF2010) or azole-resistant (TR_{34}/L98H or TR_{46}/Y121F/T289A) *A. fumigatus* strains. All compounds were added to the upper chamber of the transwell to mimic topical treatment to the lung. As shown in Figure 2a, 1 day after inoculation of azole-susceptible *A. fumigatus* NCPF2010, the control showed a GM measurement in the bottom chamber of OD=1, and PC1244 inhibited the GM level in a concentration-dependent manner. A concentration–response curve (with % inhibition) demonstrated that in this model with azole-susceptible *A. fumigatus* (Figure 2b, Table 4), voriconazole weakly inhibited the invasion (determined by GM in the endothelial compartment) but, in contrast, PC1244 and posaconazole were observed to show potent inhibition of the *A. fumigatus* penetration of the cellular bilayer. Voriconazole had no effect on the invasion of TR_{34}/L98H (Figure 2c) and TR_{46}/Y121F/T289A (Figure 2d) azole-resistant *A. fumigatus*, nor *A. flavus* up to 1 mg/L (Table 4), although both PC1244 and posaconazole demonstrated concentration-dependent inhibition of these strains' penetration through the cellular bilayer, and PC1244 was 2-fold more potent than posaconazole at inhibiting penetration by TR_{46}/Y121F/T289A *A. fumigatus* (Table 4).

In vivo antifungal activity against itraconazole-resistant A. fumigatus-infected mice

An aqueous suspension of PC1244 in isotonic saline (0.56, 2.8, 14 and 70 μg/mouse = approximately 0.028, 0.14, 0.7 and 3.5 mg/kg,

Table 2. Antifungal effects of PC1244 and known antifungal agents in azole-resistant strains of *A. fumigatus* carrying mutations in *cyp51A* gene

		PC1244				Voriconazole				Posaconazole			
Resistance mechanism	*n*	Mean[a]	MIC_{50}	MIC_{90}	range	Mean[a]	MIC_{50}	MIC_{90}	range	Mean[a]	MIC_{50}	MIC_{90}	range
CLSI													
WT	17	0.21[b]	0.25	0.35	0.125–1	0.48	0.25	16	0.25–16	0.18	0.125	0.6	0.063–1
TR_{34}/L98H	73	1.1[c]	1	2	0.25 to >8	8.7	8	>16	1 to >16	0.94	1	1	0.125 to >8
TR_{46}/Y121F/T289A	24	0.87[c]	1	1	0.25–2	>16	>16	>16	>16 to >16	0.89	1	1	0.5 to >8
G54	11	4.5[c]	>8	>8	0.125 to >8	1.4	1	4	0.5 to >16	5.5	>8	>8	0.5 to >8
M220	6	0.89[b]	1	1	0.5–1	>16	>16	>16	8 to >16	0.89	1	1	0.5–1
TR_{53}	1	1	N/A	N/A	N/A	8	N/A	N/A	N/A	2	N/A	N/A	N/A
P216S	1	0.5	N/A	N/A	N/A	1	N/A	N/A	N/A	0.5	N/A	N/A	N/A
TR_{46}[3]/Y121F/T289A	2	1	N/A	N/A	1–1	>16	N/A	N/A	>16 to >16	1	N/A	N/A	1–1
G448S	1	1	N/A	N/A	N/A	>16	N/A	N/A	N/A	1	N/A	N/A	N/A
G432C	1	1	N/A	N/A	N/A	>16	N/A	N/A	N/A	1	N/A	N/A	N/A
total (mutant only)	120	1.0[c]	1	2	0.125 to >8	15	16	>16	0.5 to >16	0.94	1	1	0.125 to >8
EUCAST													
TR_{34}/L98H[d]	8	0.16[b]	0.125	0.25	0.125–0.25	2.4	2	5.2	1–8	0.23	0.25	0.33	0.125–0.5

N/A, not applicable.
All MICs (mg/L) were read visually (and also confirmed by OD reading for EUCAST); MIC_{50} and MIC_{90} values (mg/L) represent the lowest concentrations of the compound at which 50% and 90% of the isolates are inhibited, respectively.
[a]Geometric mean. If $n=1$ or $n=2$, determined value or average of two values were provided.
[b]$P<0.05$ versus voriconazole.
[c]$P<0.01$ versus voriconazole (both with no significant difference versus posaconazole).
[d]Isolated in France.

Table 3. Antifungal effects of PC1244 and known antifungal agents in *Aspergillus* spp.

		PC1244				Voriconazole				Posaconazole			
Species	*n*	Mean[a]	MIC_{50}	MIC_{90}	range	Mean[a]	MIC_{50}	MIC_{90}	range	Mean[a]	MIC_{50}	MIC_{90}	range
A. flavus	29	0.24	0.25	0.5	0.125–0.5	0.38	0.5	0.5	0.25–0.5	0.13	0.125	0.25	0.031–0.25
A. terreus	15	0.26	0.25	0.4	0.125–0.5	0.48	0.5	0.5	0.25–0.5	0.20	0.25	0.25	0.125–0.25
A. tubingensis	15	0.33	0.25	0.5	0.125–1	0.69	0.5	1	0.25–16	0.35	0.25	0.5	0.25–1
A. nidulans	9	1	0.5	>16	0.5 to >16	0.29	0.125	16	0.125–16	0.37	0.125	>16	0.125 to >16
A. niger	6	0.18	0.19	0.25	0.125–0.25	0.32	0.25	0.5	0.25–0.5	0.22	0.25	0.25	0.125–0.25
A. nomius	5	0.44	0.5	0.5	0.25–0.5	0.76	1	1	0.5–1	0.29	0.25	0.5	0.25–0.5
A. tamarii	4	0.25	0.25	0.5	0.125–0.5	0.42	0.5	0.5	0.25–0.5	0.18	0.19	0.25	0.125–0.25

All MICs (mg/L) were read visually using the CLSI method; MIC_{50} and MIC_{90} values (mg/L) represent the lowest concentrations of the compound at which 50% and 90% of the isolates are inhibited, respectively.
[a]Geometric mean. If $n=1$ or $n=2$, determined value or average of two values were provided.

respectively) was dosed by intranasal injection once daily on days 1, 2 and 3 post-infection as the 'late intervention' regimen. PC1244 was found to inhibit fungal load in the lung (cfu) and GM in serum in a dose-dependent manner and the ID_{50} values were 13.8 μg/mouse (0.69 mg/kg) and 20.1 μg/mouse (1.01 mg/kg), respectively (Figure 3a and b). In comparison, posaconazole, at a dose of 350 μg/mouse (17.5 mg/kg), achieved only 47% inhibition of fungal load. The ID_{50} values of posaconazole were 375 μg/mouse (18.8 mg/kg) and 305 μg/mouse (15.3 mg/kg) for lung cfu and serum GM, respectively, and 27- and 15-fold higher than those of PC1244.

Discussion

We demonstrated antifungal effects of a novel inhaled triazole, PC1244, against a wide range of azole-resistant *A. fumigatus* strains,[19] which was more potent than those of voriconazole and was comparable to posaconazole in the *in vitro* systems. However, when treating temporarily neutropenic mice infected with *A. fumigatus* carrying the M220V mutation, with either PC1244 or posaconazole intranasally, PC1244 exhibited much more potent antifungal activity. This may reflect the fact that PC1244 has been optimized for a long residence time in the lung, as previously reported.[8]

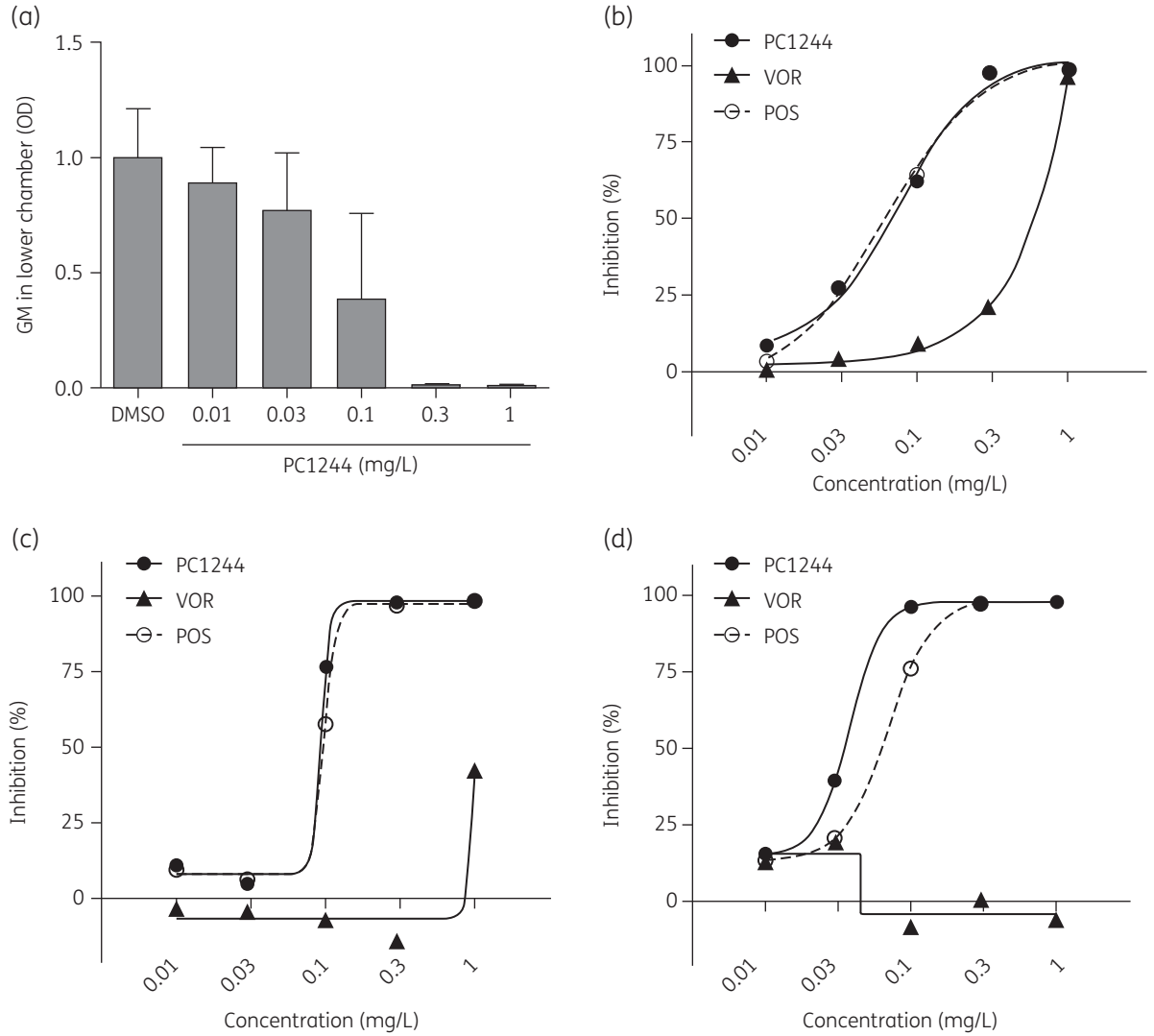

Figure 2. Efficacy of PC1244 on invasion of azole-susceptible and -resistant *A. fumigatus* in a human alveolus model. Concentration-dependent effects of PC1244 on GM (OD) in the endothelial compartment 24 h post-inoculation with azole-susceptible *A. fumigatus* NCPF2010 to the epithelial cell compartment (a). Comparison of concentration-dependent inhibitory effects of PC1244 with those of voriconazole and posaconazole on penetration of azole-susceptible *A. fumigatus* NCPF2010 (b), azole-resistant *A. fumigatus* TR_{34}/L98H (c) and TR_{46}/Y121F/T289A (d) into the endothelial compartment 24 h post-inoculation. Compounds were treated apically.

Table 4. Antifungal effects of PC1244 and known antifungal agents against azole-susceptible and -resistant strains of *A. fumigatus* and *A. flavus* in an *in vitro* model of the human alveolus

			IC_{50} (IC_{90}) values for agent indicated (mg/L)[a]		
Species	Strain number	Resistance mechanism	PC1244	voriconazole	posaconazole
A. fumigatus	NCPF2010	none	0.11 (0.13)	0.61 (0.91)	0.11 (0.15)
A. fumigatus	L98H (F)	TR_{34}/L98H	0.25 (0.38)	>3 (>3)	0.26 (0.49)
A. fumigatus	TR_{46} (H)	TR_{46}/Y121F/T289A	0.34 (0.66)	>10 (>10)	0.69 (1.27)
A. flavus	ATCC 204304	none	0.055 (0.12)	0.31 (0.58)	0.036 (0.072)

F, France (clinical); H, Himachal Pradesh, India (environmental).
[a]IC_{50} and IC_{90} values (mg/L) were determined from OD measurements of GM in the endothelial compartment.

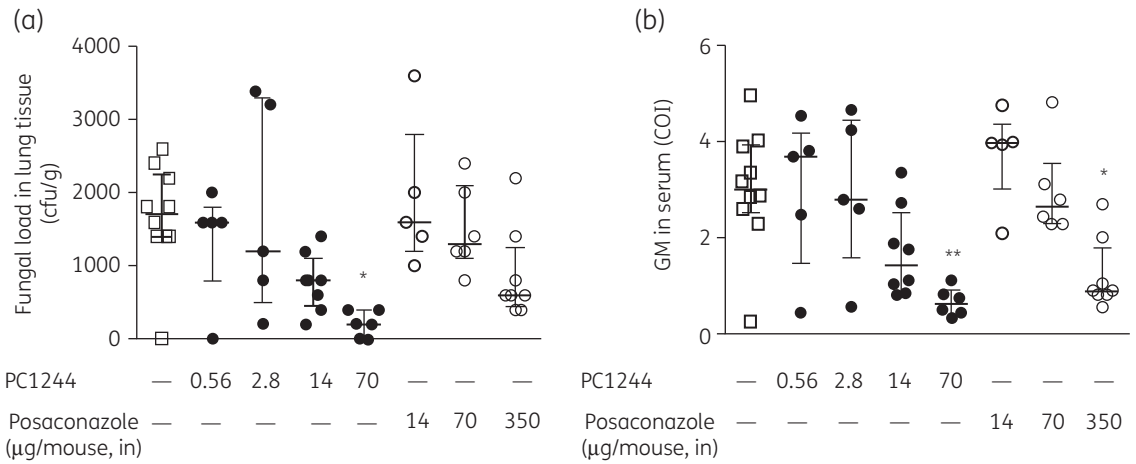

Figure 3. Antifungal activity of PC1244 and posaconazole against *A. fumigatus in vivo*. Effect of once-daily intranasal (in) treatment of PC1244 (0.56, 2.8, 14, 70 μg/mouse intranasally=approximately 0.028, 0.14, 0.7 and 3.5 mg/kg) on fungal load (cfu) in the lung tissue (a) and GM concentrations in the serum (b) of *A. fumigatus*-infected immunocompromised mice (n=5–10). Each horizontal bar was presented as median (interquartile range) from 5–10 mice per group. *P<0.01. **P<0.001. COI, cut-off index (OD in sample/OD in cut-off control provided in kit).

Azole resistance in *A. fumigatus* has emerged as a global health problem, threatening the management of diseases caused by *Aspergillus*.[5,20,21] As we have previously reported,[8] PC1244 showed greater potency against the AF91 (M220V mutation) *A. fumigatus* strains than voriconazole. These observations were confirmed in this study using a large collection of clinical isolates from India and the Netherlands (Table 2) including those with the TR_{34}/L98H and TR_{46}/Y121F/T289A mutations. PC1244 demonstrated much more potent activity than voriconazole against TR_{34}/L98H in both French (with EUCAST) and Indian/Dutch isolates (with CLSI). The activity of PC1244 (GM-MIC) was comparable to posaconazole, but strains resistant to posaconazole had low PC1244 MICs and vice versa (Figure 1d and e). Thus, resistance to PC1244 and posaconazole can apparently arise from different mechanisms, although both are CYP51A inhibitors.[8]

There are also other azole-resistant strains reported. A G448S amino acid substitution in the azole target (CYP51A) of *A. fumigatus* was identified as the cause of the resistance phenotype to voriconazole[22,23] although posaconazole retained good efficacy. Other resistant clinical and environmental isolates with promoter repeats of 53 bp (TR53) were isolated in the Netherlands and Colombia,[24,25] and $TR_{46}{}^{3}$/Y121F/T289A, with a triple 46 bp promoter repeat, causing high *cyp51A* gene expression levels, was isolated in the Netherlands.[25,26] All tested mutants with *cyp51A* promoter repeats have lower MICs of PC1244 than voriconazole.

Although microdilution tests to evaluate fungal susceptibility to antifungal drugs have been standardized for fermentative yeasts and filamentous fungi, they do not reflect a real-world, multicellular environment. IA is characterized by growth of *Aspergillus* species within the lung followed by penetration of the vascular system and infection of other host organs via the blood. Using an *in vitro* model of the human alveolus,[14,15] consisting of a bilayer of human alveolar epithelial and endothelial cells, we were able to track penetration of *A. fumigatus* into the endothelial compartment (lower chamber). GM was measured in the bottom chamber after inoculation of *A. fumigatus* into the epithelial compartment (upper chamber). PC1244 effectively inhibited the invasion of azole-resistant *A. fumigatus* (TR_{34}/L98H, TR_{46}/Y121F/T289A), where voriconazole was ineffective, which further demonstrated the strong antifungal activity of PC1244 on azole-resistant strains.

We previously reported that, when administered intranasally, PC1244 was 6- to 15-fold more potent in reducing lung fungal load and serum GM than posaconazole in temporarily neutropenic mice infected with azole-susceptible *A. fumigatus,* despite comparable MIC values *in vitro*.[8] The excellent *in vivo* effects of PC1244 were believed to be due to its persistence in bronchial cells,[8] accumulation of PC1244 after repeat dosing and its optimized properties for inhalation delivery. In this study, we have demonstrated that PC1244, when applied intranasally, exhibited 15- to 27-fold more potent antifungal activities than posaconazole against an itraconazole-resistant strain (AF91, M220V), again despite comparable MIC values in *in vitro* testing (Figure 3). Baistrocchi *et al.*[27] reported the recruitment of posaconazole-loaded granulocytes at the site of *Aspergillus* infection and demonstrated enhanced synergistic antifungal effects by exposure of *Aspergillus* to cellular posaconazole during phagocytosis. Considering the persistent action of PC1244, it is likely that granulocytes/macrophages containing PC1244 contributed to further enhancement of the antifungal effect *in vivo*. The effects might be more apparent for the itraconazole-resistant strain compared with the itraconazole-susceptible strain, as observed in our bilayer model work (Table 4, Figure 2).

The idea of delivering an antifungal agent directly to the airway or to the fungus is intuitively appealing and aims to deliver a high concentration of drug to the site of infection whilst avoiding systemic toxicity. In particular, this inhalation approach is an attractive option for delivering drug to an anastomotic site, which is poorly vascularized and hence difficult to access via a parenteral route of administration. In fact, this is an important strategy for avoiding the development of resistance during treatment to ensure that the ratio of peak concentration to MIC is adequate.[28] The frequently exploited oral and systemic routes of delivery are poor for treating airway disease, since drug concentrations achieved at the site of infection tend to be lower than those in

other, healthy organs. Delivery of antifungals directly to the lung enables high AUC/MIC ratios to be achieved locally, reducing the risk of resistance development. The benefits of inhaled administration for the treatment of invasive pulmonary aspergillosis has also been shown in numerous studies involving amphotericin B, itraconazole and voriconazole.[29–31] However, these repurposed compounds were not optimized for inhalation therapy so consequently had shorter-than-ideal lung residence times.

This study had several limitations. Firstly, we used two different methods (EUCAST and CLSI) of broth microdilution, which might cause difficulties in direct comparison of the data. The EUCAST method was used for assays performed in Europe and the CLSI method for those performed in India. For the TR34-L98H strain shown in Table 2, no substantial difference in the geometric mean MIC has been observed although French strains (EUCAST) seem to be more susceptible to azoles than Indian TR34-L98H strains. For bridging, we tested three strains (NCPF2010, TR46/Y121F/T289A from Himachal, India, TR34-L98H from France) using both EUCAST and CLSI, and we did not observe a big difference between the MICs by each method [PC1244 MIC in EUCAST and CLSI, respectively: NCPF2010 (0.031 and 0.016 mg/L); TR34-L98H (France) (0.125 and 0.125 mg/L); TR46/Y121F/T289A (Himachal) (0.25 and 0.125 mg/L)].

Secondly, we compared the *in vivo* performance of PC1244 with posaconazole, which has not been optimized for topical treatment and its physicochemical properties are not identical to that of PC1244. However, the data shown in Table S1 demonstrate that PC1244 is less aqueous-soluble than posaconazole, so both were dosed as aqueous suspensions rather than solutions. Neither posaconazole nor PC1244 are Lipinski-compliant, but have similar molecular weights, topological polar surface area (TPSA) and log *P* values, and are both markedly different from voriconazole. Thus, posaconazole and PC1244 possess similar physicochemical properties. As previously reported,[8] preliminary studies using non-infected mice dosed intratracheally with 40 μL of a 2 mg/mL aqueous suspension of PC1244, plasma concentrations ranged between 41.5 and 50.7 ng/mL 24 h post-dose despite decent *in vivo* effects after once-daily treatment, which were similar to those for posaconazole (plasma: 15.6–125 ng/mL 24 h post-dose), although the *in vivo* activity of PC1244 was superior to that of posaconazole. As PC1244 has much less oral availability than posaconazole (Pulmocide Ltd, unpublished data), the exposure results from absorption through the respiratory tract (not by accidental ingestion of compound during dosing). Overall, we do not, therefore, believe that the different water solubility of the compound is a viable explanation for the superior performance of PC1244 relative to posaconazole. As discussed above, PC1244 has been optimized for a long residence time in the lung and this property is a key component for superior *in vivo* performance. Further studies to identify the molecular mechanism of longer lung residency is required.

Thirdly, this model is unlike a natural infection setting. Patients who are infected naturally are likely to be treated later in the course of disease, with greater disease severity. In addition, patients might be treated by inhalation as PC1244 is designed to be delivered to the lung via inhalation. Therefore, it may be inappropriate to directly extrapolate the results of the study to a clinical setting. However, based on this result, prophylaxis or empirical treatment (even intranasally) will be an option for PC1244.

Fourthly, as PC1244 has not been dosed to humans as yet, we don't have any data to demonstrate that the concentrations shown in this study could be achievable in humans. However, we have some data to support that the concentrations would be achievable in humans, based on our *in vivo* animal studies and experiences with our first development candidate PC945 (Phase 2a),[8,32] which is an inhaled medicine with similar physicochemical properties to PC1244. In preclinical safety studies with PC945, the lung:plasma concentration ratio was found to be ~7000. As previously published,[8] in non-infected mice dosed intratracheally (40 μL of a 2 mg/mL aqueous suspension of PC1244), plasma concentrations of PC1244 ranged from 111 to 303 ng/mL 2 h post-dose and from 249 to 339 ng/mL 8 h post-dose. If PC1244 were to show similar properties to PC945, the lung concentrations achieved should comfortably exceed those required to deliver the activity described in this study. Further preclinical pharmacokinetic and clinical studies will be required to clarify this.

Taken together, PC1244 therefore has the potential to be developed as a new topical antifungal for the treatment of pulmonary azole-resistant *A. fumigatus* infections.

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Transparency declarations

K. I., P. S. and G. R. are employees of Pulmocide Ltd and (co)-founders of Pulmocide Ltd. K. I. and G. R. retain an honorary contract with Imperial College. T. C. and L. D. are employees of Pulmocide Ltd. J. F. M. received grants from Astellas, Basilea and Pulmocide and has been a consultant to Astellas, Basilea, Scynexis and Merck and received speaker's fees from Merck, United Medical, TEVA and Gilead Sciences. All other authors: none to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

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