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Antifungal Effects of PC945, a Novel Inhaled Triazole, on *Candida albicans* Pulmonary Infection in Immunocompromised Mice

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Abstract

Candida species (spp.) are frequently detected in fungal cultures of respiratory secretions, and there is growing evidence that *Candida spp*. are involved in the pathogenesis of respiratory diseases. The aim of this study is to investigate *in vivo* effects of a novel antifungal triazole, PC945, optimized for topical delivery, in *Candida albicans* pulmonary infection murine model as well as to evaluate *in vitro* antifungal profiles of PC945 with an extended collection of *Candida spp*. In temporarily neutropenic, immunocompromised mice, intranasal inoculation with *C. albicans* (529L) caused significant pulmonary inflammation and minor acute lung injury as well as high local *Candida* burden. PC945 saline suspension, dosed intranasally once daily, starting one day post *Candida* inoculation, dose-dependently (0.56~14 μg/mouse) improved survival rate and inhibited fungal load in the lung on Day 5 post inoculation as well as lung inflammation. These effects by PC945 were 7~25-fold more potent than those of voriconazole, despite being of similar *in vitro* antifungal activity versus this strain. Furthermore, extended prophylaxis with low dose PC945 (0.56 μg/mouse) was found to inhibit fungal load more potently than the shorter treatment regimens, suggesting antifungal effects of PC945 accumulated on repeat dosing. In addition, antifungal susceptibility testing on 88 *Candida* isolates *(C. albicans, C. parapsilosis, C. tropicalis, C. lucitaniae, C. glabrata, C. guilliermondii)* revealed that PC945 has potent effects on *Candida* species broadly. Thus, PC945 has the potential to be a novel topical therapy for the treatment of *C. albicans* pulmonary infection in humans.

Keywords: Candida, Lung, Intranasal, Antifungal, Acute lung injury, Triazole, Mice, Prophylaxis

Abbreviations: ATCC: The American Type Culture Collection; BALF: Bronchoalveolar Lavage Fluid; CFU: Colony Forming Unit; CLSI: Clinical and Laboratory Standards Institute; CXCL1: C-X-C motif Chemokine Ligand 1; CYP51A1: Lanosterol 14α-demethylase; DMSO: Dimethyl Sulfoxide; EUCAST: European Committee on Antimicrobial Susceptibility Testing; FEV1: Forced Expiratory Volume; Geo-mean: Geometric Mean; IN: Intranasal; MIC_{50}: Minimum Inhibitory Concentration required to inhibit the growth of 50% of fungal isolates tested; MIC_{90}: Minimum Inhibitory Concentration required to inhibit the growth of 90% of fungal isolates tested; TNFα: Tumor Necrotic Factor α

Introduction

Candida spp. are often detected from fungal cultures or mycobiome analysis of respiratory secretions, but are not usually treated since, even if detected persistently, the presence is assumed to be benign colonization unless invasive candidiasis in deeply immunocompromised subjects is suspected. Thus, although antifungal therapy is standard treatment for *Candida* infections observed in other organs such as the vagina and skin, no practical guidelines to treat *Candida* infection in the respiratory tract, except for invasive candidiasis, have been developed [1,2].

However, there is growing evidence that *Candida spp*. may be causally involved in chronic respiratory diseases as well as invasive candidiasis via the respiratory tract. For example, *Candida albicans* (*C. albicans*) as well as *Aspergillus fumigatus* are often detected in patients with non-cystic fibrosis bronchiectasis, who show compromised mucociliary clearance [3,4]. *Candida* can also cause pneumonia with a severe clinical course [5,6]. Johnson reported 11 cases of chronic *Candida* bronchitis patients who had persistent sputum production despite antibiotic treatment, where antifungal therapy led to a good or excellent clinical response (improved respiratory symptoms and sputum production) within 3 weeks [2]. Corticosteroid treatment is known to facilitate *C. albicans* colonization of the respiratory tract [7] as well as oral/esophageal candidiasis [8]. *Candida* itself also causes allergic bronchial pulmonary mycosis [9] and sensitization to *C. albicans*, while not an aeroallergen, is seen in up to 10% of individuals with mild stable asthma and 33% of patients with severe asthma [10]. As well as worsened sputum production and bronchitis, persistent *C. albicans* was associated with worse post-bronchodilator forced expiratory volume (FEV1), more frequent bronchiectasis and more hospital-treated exacerbations in non-cystic fibrosis patients with bronchiectasis [11]. Furthermore, *Candida* airway colonization has been shown to be associated with prolonged duration of mechanical ventilation and ICU/hospital length of stay. The overall hospital mortality in this group was significantly higher than patients with non-*C. albicans* fungal infections [12,13]. However, most reports of *Candida* infection of the respiratory tract are still on a case-reportable basis and no placebo-controlled intervention study on *Candida* infection was reported.

Beta-glucan, mannan and chitin, components of the yeast cell wall, may act as proinflammatory factors in the lung [14]. In addition, *Candida* is known to interplay with both Gram-positive and Gram-negative bacteria in the environmental biofilm, through quorum-sensing molecules [15]. *In vivo*, *C. albicans* infection was found to worsen pneumonia caused by *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* in rats, enhancing the production of inflammatory cytokines in the lung, and inhibiting phagocytosis by alveolar macrophage [16-18].

Systemic triazole therapy is the basis of treating infections with pathogenic fungi but the adverse effects of itraconazole, voriconazole and posaconazole are well characterized and thought to be a consequence of their pharmacological effects in host tissues [19,20]. 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 [21,22]. In addition, fungus colonization occurs in pre-existing lung cavities due to tuberculosis or on airway surfaces where fungi first deposit and grow, and is difficult to deliver and maintain appropriate local levels of anti-fungal agents after oral treatment [23]. Furthermore, resistance to antimicrobial agents is an emerging problem worldwide. Although high levels of, and continuous exposure to, antimicrobial agents are known to decrease the risk of mutation induction leading to resistance in bacteria [24], systemic treatment hardly achieves continuous high levels of exposure (for example, AUC/MIC, >60 [24]) in lung cavities in all patients although the level might exceed MIC values [23,25]. Thus, inhaled treatment has several potential advantages versus oral/systemic treatment which alter the risk benefit ratio of treatment favorably. Tolman and colleagues have demonstrated that prophylaxis with an aerosolized aqueous intravenous formulation of voriconazole significantly improved survival and limited the extent of invasive disease, as assessed by histopathology, in an invasive pulmonary murine model [26]. In human studies, aerosolization of voriconazole showed beneficial effects but it required frequent and high doses as voriconazole was not optimized as an inhaled medicine [27,28].

PC945, 4-[4-(4-{[(3R,5R)-5-(2,4-difluorophenyl)-5-(1H-1,2,4-triazol-1-ylmethyl) oxolan-3-yl] methoxy} c-3-methylphenyl) piperazin-1-yl]-N-(4-fluorophenyl) benzamide, is a novel antifungal triazole [29-32], which has been optimized for inhaled delivery [33], maximizing the potential for therapeutic activity in the lungs while minimizing the potential for toxicity in other organs. PC945 has been shown to be an inhibitor of the enzyme lanosterol 14α-demethylase (CYP51A1) and have potent *in vitro* antifungal activity against *Aspergillus spp*., *Candida spp*. including *C. auris* and other fungi [32,34]. Topical once daily treatment of PC945 was also found to be highly effective in temporarily neutropenic mice infected with *A. fumigatus* when compared with intranasally treated posaconazole and voriconazole. Interestingly, the first case report describing the successful use of PC945 to treat a refractory *Aspergillus* bronchial anastomotic infection and tracheobronchitis in a lung transplant on top of standard care has recently been published [35]. PC945 was also well tolerated in healthy subjects and mild asthmatics in the first-in-human study [36].

The aim of this study is to establish *C. albicans* pulmonary infection murine model to investigate the *in vivo* antifungal effects of intranasally dosed PC945 and also evaluate *in vitro* antifungal effects of PC945 by broth microdilution assay with an extended collection of *Candida spp*.

Materials and Methods

Antifungal agents

PC945 was synthesized by Sygnature Discovery Ltd. (Nottingham, UK), while voriconazole (Tokyo Chemical Industry UK Ltd., Oxford, UK) and posaconazole (Apichem Chemical Technology Co., Ltd., Zhejiang, China) 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 test agents were suspended in physiological saline.

In vivo antifungal activity against *C. albicans* infection

Specific pathogen-free A/J mice (male, 5 weeks old) were purchased from Sankyo Labs Service Co. Ltd. (Tokyo, Japan) and adapted for 1 week in a temperature (24 ± 1°C) and humidity (55 ± 5%) controlled room, under a 12-h day-night cycle. The mice were reared on a standard diet and tap water *ad libitum*. A/J mice were used for *A. fumigatus* infection and proved to be more efficiently infected as described previously [37]. Animals 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 as previously reported [37-40]. Both hydrocortisone and cyclophosphamide were diluted with physiological saline. To avoid bacterial infection, drinking water was supplemented with tetracycline hydrochloride (Sigma T7660; 1 µg/ml) and ciprofloxacin (Fluka 17850; 64 µg/ml). *C. albicans* (ATCC MYA4901 [529L]), purchased from the American Type Culture Collection (Manassas, VA, USA), was grown on Sabouraud dextrose agar (Difco Laboratories Ltd., West Molesey, UK) plates for 3 days at 35°C. Fungal colonies were aseptically dislodged from the agar plates and suspended in physiological saline. On the day of infection, yeast counts were assessed by hemocytometer, and the inoculum was adjusted to obtain concentrations of 5×10^7 /mL of sterile physiological saline. On Day 0, the suspension was administered intranasally (total volume 50 µL/mouse; 25 µL per each nostril), so 2.5×10^6 yeast was inoculated per mouse.

PC945 or voriconazole, suspended in physiological saline, were administered intranasally (total volume 35 µL/mouse; approximately 17.5 µL each to each nostril) once daily from Day 1 to Day 5 post inoculation as shown in Figure 1. To investigate extended prophylaxis, PC945 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 changed daily, especially after infection, the accurate dose unit was µg/mouse. However, we also calculated estimated dose as mg/kg, assuming the average body weight of 20g just before treatment and 60% exposure after intranasal dosing. Therefore, 35 µl injections of 0.016, 0.08, 0.4, 2, 10 mg/ml were equivalent to 0.56, 2.8, 14, 70, 350 µg/mouse, respectively, corresponding to approximately 0.028, 0.14, 0.7, 3.5 and 17.5 mg/kg, respectively (Table S1). To reduce suffering and distress during intranasal injection, local anesthesia (3% isoflurane) was applied intranasally before the procedure.

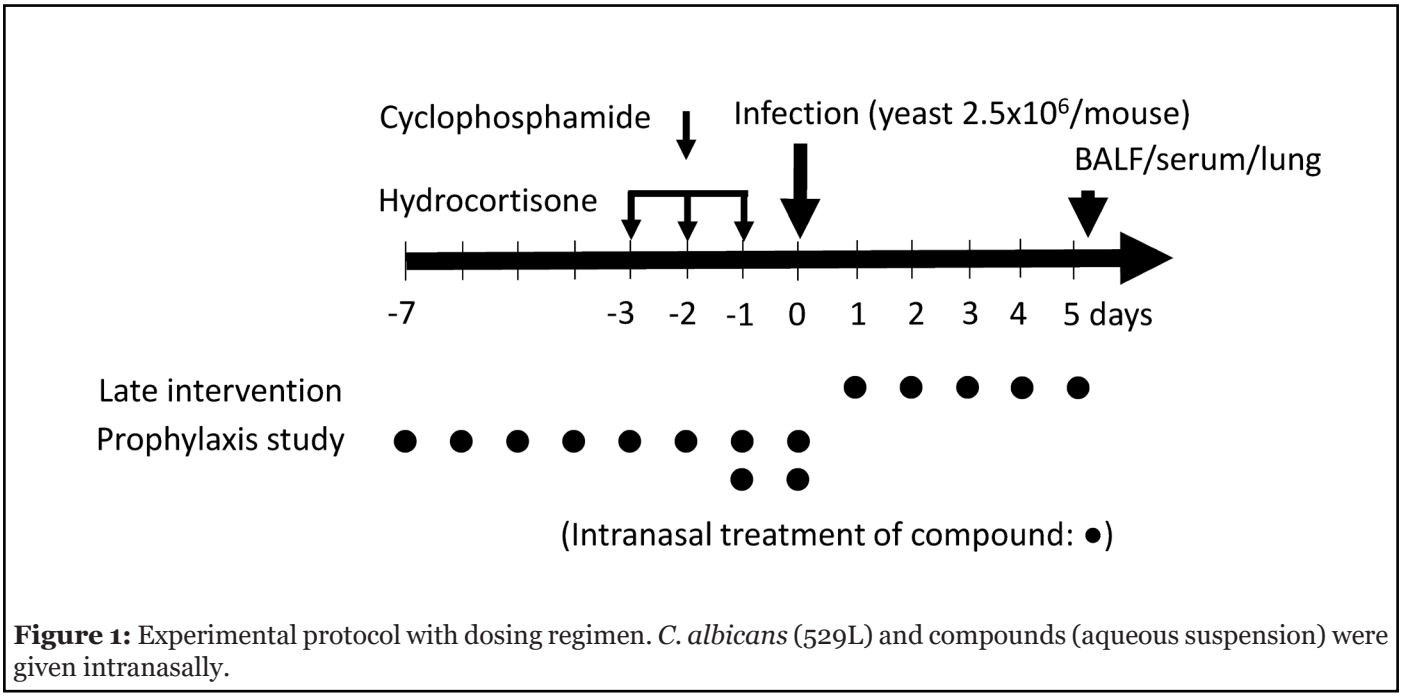

Figure 1: Experimental protocol with dosing regimen. *C. albicans* (529L) and compounds (aqueous suspension) were given intranasally.

Deaths, and physical condition and the body weights of surviving animals were monitored and recorded daily for 5 days. A body weight loss of more than 20%, compared with an animal's weight when the experiment was commenced, or any serious condition (high or low body temperature, severe dyspnea, ruffled coat, lethargy) were defined as "drop-out" events (as a humane endpoint), and animals were euthanized by an intraperitoneal injection with high dose of pentobarbital (100 mg/kg) as soon as possible when the animals reached endpoint criteria. Bronchoalveolar lavage fluid (BALF) was collected through cannulated tracheas using physiological saline [41] on day 5 post infection (6 hrs. after the final treatment) or on the day that the mouse dropped out of the study. Blood was then collected via cardiac puncture except for dead mice, and left lung tissue was removed to prepare a tissue homogenate. All animal studies were approved by the Ethics Review Committee for Animal Experimentation of Nihon University. *C. albicans* studies were approved by the Microbial Safety Management Committee of Nihon University School of Pharmacy (E-H25-001). Animal handling training was provided to the staffs in the University before starting animal work.

Biomarker analysis

For semi-quantitative tissue fungal load analysis, 100 mg of lung tissue (left lung) was removed aseptically and homogenized in 1.0 mL of sterile saline and kept on ice to limit fungal growth of in homogenates until all samples were processed. Homogenates were prepared using a mini cordless CG-4A homogenizer (Funakoshi Ltd., Tokyo, Japan) using mild conditions (2 repeated cycles of 10 seconds homogenization and 2 min resting on ice) before centrifugation at 1400 × g for 1 min (room temperature). The supernatants obtained were serially diluted using sterile physiological saline and plated on Sabouraud agar plates (50 μL/plate) as soon as possible, and then incubated at 35 ± 1°C for 72 to 96 hrs. The colonies of *C. albicans* on each plate were counted and corrected to the dilution factor, and the fungal titer is presented here as CFUs per gram of lung tissue. The levels of CXCL1 in BALF and TNFα in serum were determined using Quantikine® mouse ELISA kits (R&D systems, Inc., Minneapolis, MN, USA). The *Candida* β-glucan concentration in BALF or serum was determined with Glucatell kits (Associates of Cape Cod, Inc., Massachusetts, USA). For histology, the right lung was fully inflated by intratracheal perfusion with 10% paraformaldehyde in PBS. Lungs were then dissected free and placed in fresh paraformaldehyde solution. Routine histological techniques were used to paraffin-embed the tissues, and 4 μm sections of whole lung were stained with either hematoxylin and eosin or Grocott stain (Silver stain kit, Sigma HT100A).

***Candida* Strains and MIC determination**

Antifungal susceptibility testing for 88 *Candida* isolates *(C. albicans* [37]*, C. parapsilosis* [17]*, C. tropicalis* [7]*, C. lucitaniae* [11]*, C. glabrata* [10]*, C. guilliermondii* [6]*)* was performed in accordance with the guidelines in the Clinical and Laboratory Standards Institute (CLSI) M27-A4 document (4th edition) [42] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) definitive document E.DEF 7.3 Method [43] at Evotec (UK) Ltd (Manchester, UK). For testing by CLSI methodology, as per protocol, RPMI-1640 medium with 3-(*N*-morpholino) propanesulfonic acid (RPMI), an inoculum of 0.25~0.5 × 10^3 CFU/well, and incubation at 35°C for 24h in ambient air was used. The results were read after 24-h incubation. MIC endpoints were determined visually as the lowest concentration of compound that resulted in a decrease of growth by ≥50% relative to that of the growth control (azole endpoint) [42]. Stock solutions of test agents were prepared in neat DMSO and then diluted 100-fold to the desired concentrations with DMSO. The compound DMSO solution was applied to fungus growth media to ensure that the final concentration of DMSO was 1% (v/v) in all plates evaluated. For EUCAST, suspensions equivalent to a McFarland 0.5 standard were prepared for each test strain in 5 mL sterile water and diluted 1:10 in sterile water to provide an inoculum of 1-5 × 10^5 CFU/mL. 100 μL of each inoculum was dispensed into all wells, except negative control wells (100 μL sterile water added), to provide 0.5-2.5 × 10^5 CFU/well and a final volume of 200 μL single-strength RPMI-1640 was added per well. Assay plates were incubated according to EUCAST E.DEF 7.3 guidelines (35 ± 2°C for 24 ± 2 h in ambient air). An inoculum purity and viability check of the QC strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 was performed on SABC agar. Compound preparation and treatment were conducted as shown above. The MIC was read as the lowest drug concentration that prevented any discernible growth (50% inhibition).

Statistical analysis

For *in vitro* test data, statistical analyses of all the data were performed using the PRISM 8® software program (GraphPad Software Inc., San Diego, CA, USA)., and the results are expressed as geo-metric mean (geo-mean), MIC_{50} (50 percentile) and MIC_{90} (90 percentile). Multiple comparison was performed by ANOVA followed by Turkey's multiple comparison test. Statistical significance was defined as $p<0.05$.

For *in vivo* test data, survival analysis was performed by Kaplan-Meier plots followed by the log rank (Mantel-Cox) tests using the PRISM 8® software program (GraphPad Software Inc., San Diego, CA, USA). All biomarker results are expressed as means ± standard error of the mean (SEM). CFU result was expressed as geometric means ± standard error (SD). Multiple comparison was performed

by the Kruskal-Wallis with Dunn's *post hoc* comparison using the PRISM 8® software program (GraphPad Software Inc., San Diego, CA, USA). Comparison between 2 groups was performed by paired Wilcoxon paired rank test for MIC analysis or unpaired Mann-Whitney test *in vivo*. The $ID_{3\text{-log}}$ value, which is the dose to reduce fungal load at 3-Log, was also calculated from dose-response curve using a nonlinear regression analysis with three parameter fitting using the PRISM 8® software program. Statistical significance was defined as $P<0.05$.

Results

Fungal and proinflammatory biomarker analysis in *Candida albicans* inoculated immunocompromised mice

Intranasal inoculation of *C. albicans* (529L [ATCC®MYA4901™] strain) with either 2.5×10^6/mouse or at 1.0×10^7/mouse showed remarkable fungal loads in the lung and concentrations of biomarkers in BALF on Day 3 and 5 post inoculation (Figure 2). β-glucan in serum (as a marker of *Candida* infection) reached a peak on Day 5 at the lower inoculum, 2 days after the peak of fungal burden in the lung (Figure 2b). However, β-glucan was lower in mice inoculated with higher yeast dose, suggested pathogenesis at higher inoculation locates in the lung dominantly rather than systemic dissemination. In addition, there were big viabilities of the levels of β-glucan among mice, and this might be not a robust biomarker in this model. CXCL1 in BALF was increased on Day 3 (Figure 2c), and TNFα in serum peaked 2 days later (Figure 2d), which showed similar kinetics with serum β-glucan. Thus, there is a time-gap between lung infection and subsequent systemic disseminated *Candida* infection at lower inoculum. Considering biomarker data viability and loss of animals from the study, we decided to use 2.5×10^6/mouse inoculum and observe for 5 days, so

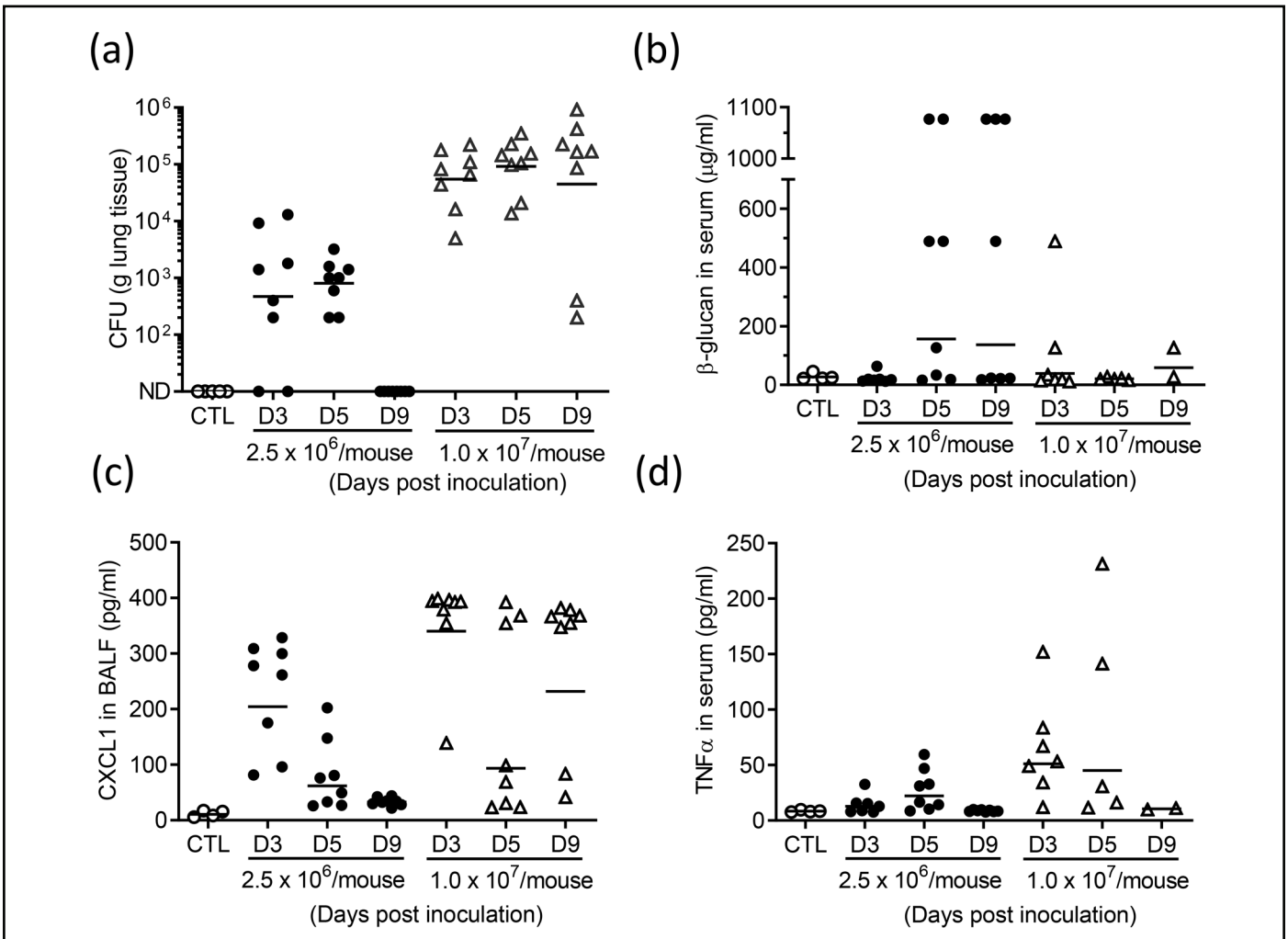

Figure 2: Time-dependent changes of fungal load in the lung (a), β-glucan in serum (b), CXCL1 in BALF (c) and TNFα in serum (d) in immunocompromised mice inoculated intranasally with *C. albicans* at 2.5×10^6 yeasts/mouse or at 1.0×10^7 yeasts/mouse. CTL: non-infected immunocompromised control mice; ND: not detectable.

that both survival and biomarkers could be analyzed for pharmacological evaluation of PC945.

Therapeutic antifungal effects of intranasal PC945 on *Candida albicans* infected mice

Prior to *in vivo* study, we evaluated the anti-fungal activities of PC945 and voriconazole against *C. albicans* 529L, which was used for *in vivo* assay. In the broth microdilution assay [EUCAST method], PC945 showed an MIC value of 0.016 μg/mL, whereas the MIC for voriconazole was 0.008 μg/mL, suggesting that the *in vitro* effect of voriconazole was similar to that of PC945 against this strain.

As shown in Figure 1, PC945 was given intranasally daily a day post intranasal *Candida albicans* inoculation in immunocompromised, temporarily neutropenic mice. In this model, 79% (11/14) of control mice died or had dropped out of the study by day 5 post infection, so a minority (21%) of mice survived (Table 1, Figure 3a). Intranasally dosed PC945 saline suspension showed a dose-dependent improvement in survival rate (21, 36 and 64% at doses of 0.56, 2.8 and 14 μg/mouse, respectively) where the effect at the top dose was statistically significant (Table 1, Figure 3a). However, although intranasally dosed voriconazole also improved survival rate dose-dependently (Table 1), significant improvement was seen only at the highest dose (350 μg/mouse), 25-fold higher than the

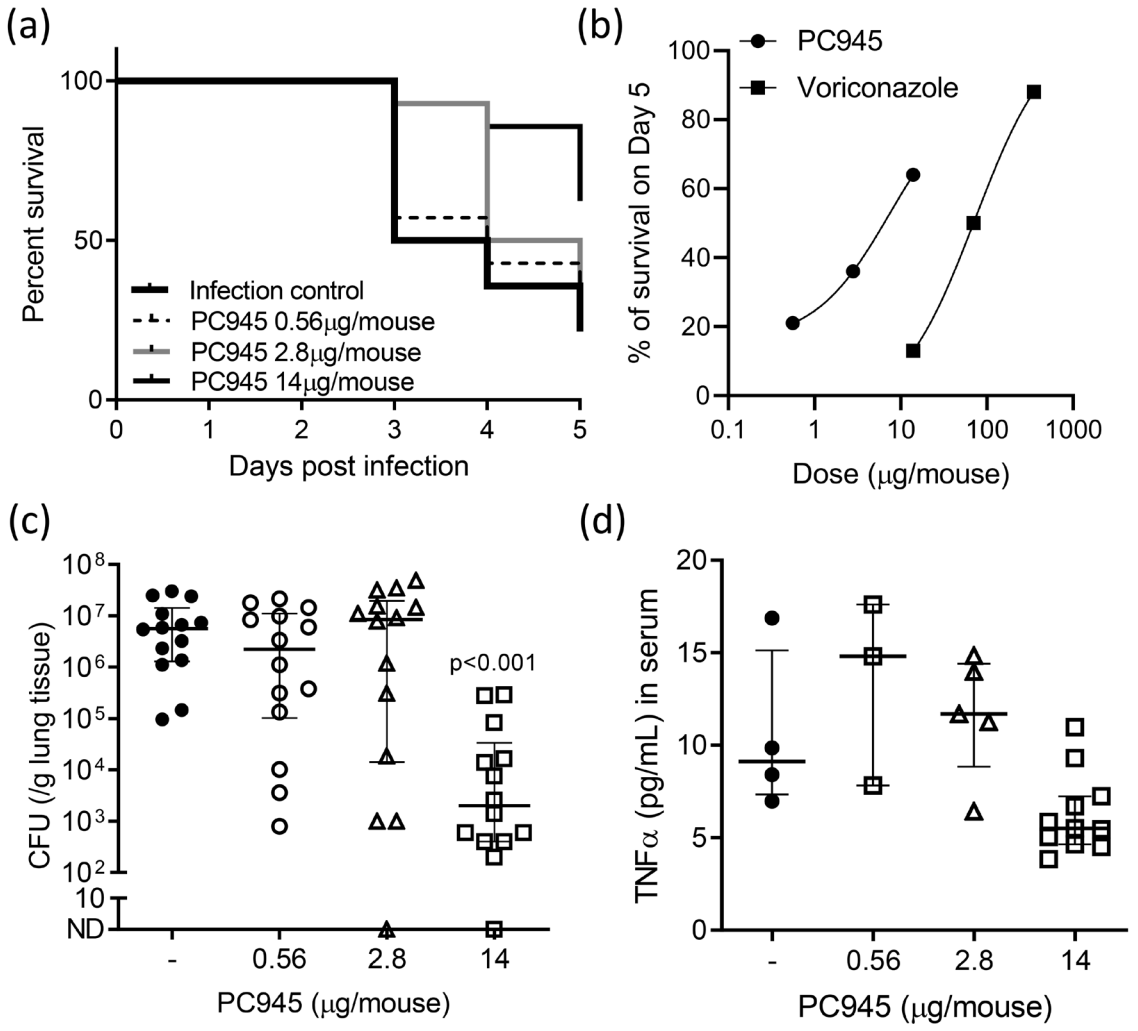

Figure 3: Antifungal activity of PC945 against *C. albicans in vivo*. Effect of once daily intranasal treatment of PC945 saline suspension (0.56, 2.8, 14 μg/mouse) for 5 days on survival in *C. albicans* infected immunocompromised mice (N=14) (a) and a dose-response curve of survival rate for PC945 (0.56, 2.8, 14 μg/mouse) and voriconazole (14, 70, 350 μg/mouse) (b). Lung fungal loads (CFU/g lung tissue) in all mice (c) and TNFα in serum in survived mice (d) were evaluated. Antifungal activity of extended prophylaxis treatment of PC945 was also evaluated. Each horizontal bar shows the median with interquartile range.

Test Agent	Dose (μg/mouse)	Death n/N	Drop-out n/N	Survivor n/N (%)	Median survival day	Log-rank (Mantel-Cox Test) p-value
Vehicle	-	10/14	1/14	3/14 (21)	3.5	-
PC945	0.56	10/14	1/14	3/14 (21)	4.5	0.91
	2.8	8/14	1/14	5/14 (36)	5.0	0.34
	14	3/14	2/14	9/14 (64)	undefined	0.0065
Vehicle	-	4/8	3/8	1/8 (13)	4.0	-
Voriconazole	14	5/8	2/8	1/8 (13)	4.0	0.99
	70	3/8	1/8	4/8 (50)	5.0	0.055
	350	0/8	1/8	7/8 (88)	undefined	0.0018

Table 1: *In vivo* activities of effects of PC945 and voriconazole on in vivo survival on Day5 post *C. albicans* inoculation in immunocompromised mice; n: number; N: total.

dose of PC945, which achieved statistically significant improvement. When 50% survival improvement dose is calculated, PC945 (6.4 μg/mouse) was 11-fold more potent than voriconazole (70.0 μg/mouse) as shown in Figure 3b. Thus, PC945 was more effective than voriconazole in the *in vivo* system when dosed intranasally.

In this model, high levels of fungal burden in lung (geometric mean: 6.6 ± 0.20 Log CFU/g of lung) were observed on day 5 in control mice, which PC945 dose-dependently inhibited as seen in Figure 3c. The dose which reduced fungal load by 3 Logs was 13.3 μg/mouse and 91.0 μg/mouse for PC945 and voriconazole, respectively, suggesting PC945 was 6.8-fold more potent than voriconazole. In addition, high levels of the inflammatory marker CXCL1 (5.3 ± 1.1 ng/mL) were detected in BALF on day 5 in control mice, which was significantly inhibited by PC945 at 14 μg/mouse (Table 2). Serum TNFα was only evaluated in surviving mice, and PC945 showed a trend towards dose-dependent reduction (Table 2 and Figure 3d), which was not statistically significant due to the smaller sample size (Table 2). Histology revealed significant inflammation and minor acute lung injury as well as *Candida* colonization (Grocott staining) on day 5 post infection or earlier (mortality) in immunocompromised control mice. PC945 completely eliminated *Candida* from lung at 14 μg/mouse, except for animals where there was early death (Figure 4a and b).

Treatment	Dose (μg/mouse)		CFU in lung (Log, CFU/g)	CXCL1 in BALF (ng/mL)		TNFα in serum (pg/mL)
		N	[log reduction]	[% inhibition]	N	[% inhibition]
Vehicle + yeast	None	14	6.6 ± 0.20	5.3 ± 1.1	4	11 ± 2.2
PC945	0.56	14	5.8 ± 0.39 [0.7]	5.6 ± 0.93 [-7]	3	13 ± 2.9 [-28]
	2.8	14	5.7 ± 0.62 [0.9]	4.8 ± 1.1 [9]	5	12 ± 1.5 [-11]
	14	14	3.4 ± 0.39 *** [3.1]	2.0 ± 1.0* [62]	11	6.3 ± 0.65 [40]
Vehicle + yeast	None	8	7.0 ± 0.28	3.3 ± 0.90	3	12 ± 3.2
Voriconazole	14	8	6.8 ± 0.29 [0.1]	3.0 ± 0.92 [9]	3	9.3 ± 1.4 [20]
	70	8	4.5 ± 0.49* [2.5]	1.6 ± 0.75 [52]	5	7.7 ± 1.2 [34]
	350	8	1.3 ± 0.66*** [5.6]	0.096 ± 0.0077** [97]	8	8.1 ± 0.72 [30]

BALF: Bronchoalveolar Lavage Fluid; CFU: Colony Forming Unit, N: Number; The data are shown as the mean ± standard error of mean (SEM); N=3-14; Percentage inhibition with respect to vehicle; *p<0.05, **p<0.01, ***p<0.001; vehicle control, Kruskal-Wallis test, Dunn's multiple comparisons test.

Table 2: Effects of PC945 and voriconazole on biomarkers in BALF and serum obtained from *C. albicans* infected immunocompromised mice.

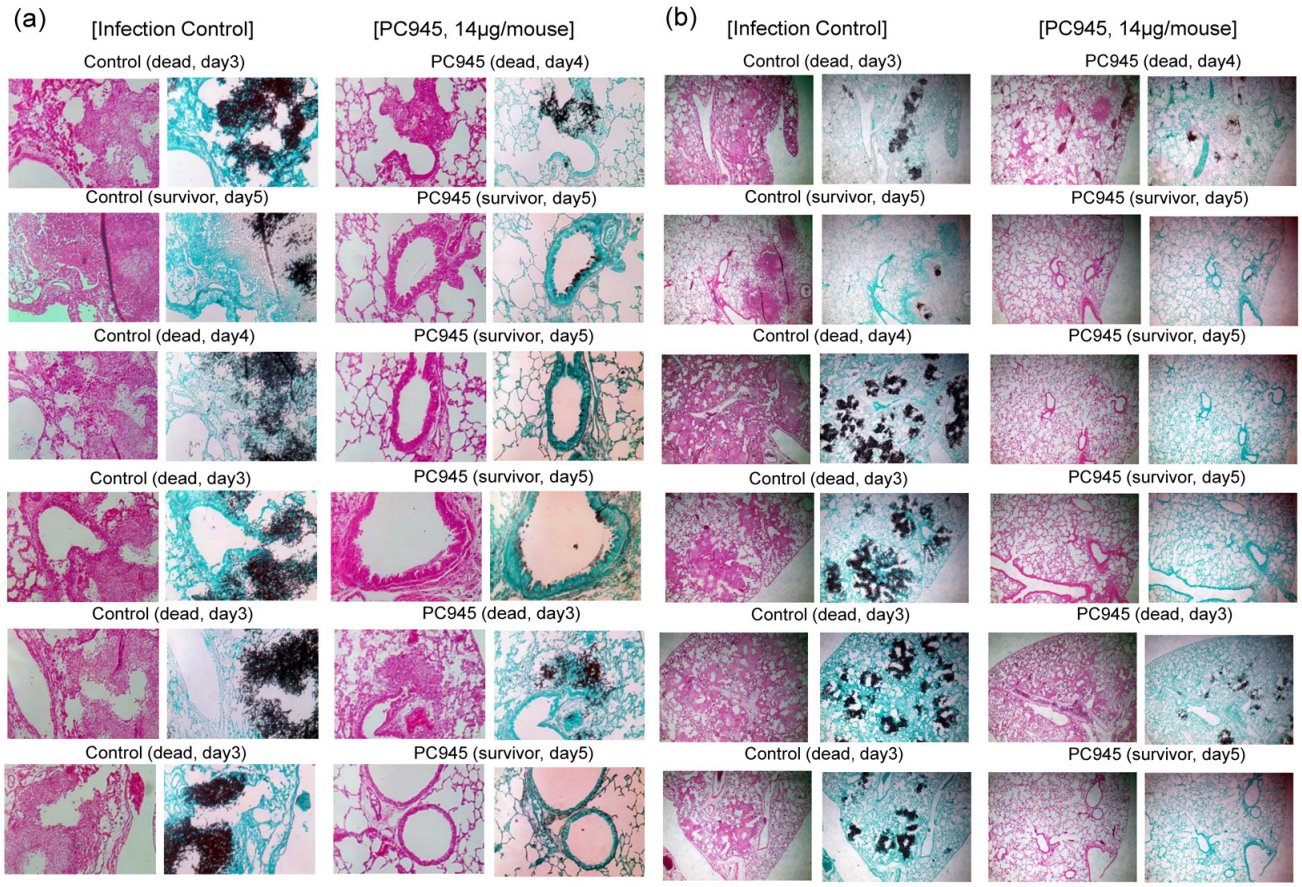

Figure 4: Histology of lung obtained from control and PC945-treated *C. albicans* infected immunocompromised mice. PC945 (14 μg/mouse) or saline was treated intranasally once daily, and some mice were dead before Day 5 post *C. albicans* inoculation and others were sacrificed on Day 5, 6 hrs. after the last dose. Lung specimens were stained with Hematoxylin eosin (H&E) and Grocott fungus staining agent (×200 (panel a) and ×40 (panel b)).

Antifungal effects of extended prophylaxis with intranasal PC945 on *Candida albicans* infected mice

As well as therapeutic dosing, the anti-fungal potential of PC945 using extended prophylaxis was evaluated (Figure 1). Extended prophylaxis (Day -7 to Day 0) at 0.56 μg/mouse showed marked and significant inhibition of fungal load in lung tissue (CFU) compared with shorter prophylaxis (Day -1 to Day 0) (Figure 5a). These effects were equivalent to those observed by therapeutic treatment of PC945 at 14 μg/mouse (Figure 3), suggesting extended prophylaxis was 25-fold more effective than therapeutic treatment. The extended prophylactic treatment also showed significant reduction of serum TNFα (Figure 5b).

In vitro antifungal activity against *Candida spp.*

The geometric mean MIC, MIC_{50} and MIC_{90} of PC945 against all *Candida* spp. tested by CLSI method were 0.027, 0.031 and 0.25 μg/mL, respectively, >2-fold weaker than voriconazole (≤0.016, ≤0.016, 0.063) (Table 3) and at least 2-fold more potent than posaconazole (0.097, 0.063, 0.5) (Table S2). When analyzing each *Candida* species separately, geometric mean MIC values of PC945 against *C. albicans* [37 isolates], *C. tropicalis* [7], *C. parapsilosis* [17], *C. glabrata* [10], *C. lucitaniae* [11] and *C. guilliermondii* [6] were 0.017, 0.063, 0.017, 0.12, ≤0.016 and 0.18 μg/mL. The MIC_{90} on *C. tropicalis* and *C. guilliermondii* for PC945 and other antifungals are relatively higher because of limited isolate number tested. PC945 was therefore equal

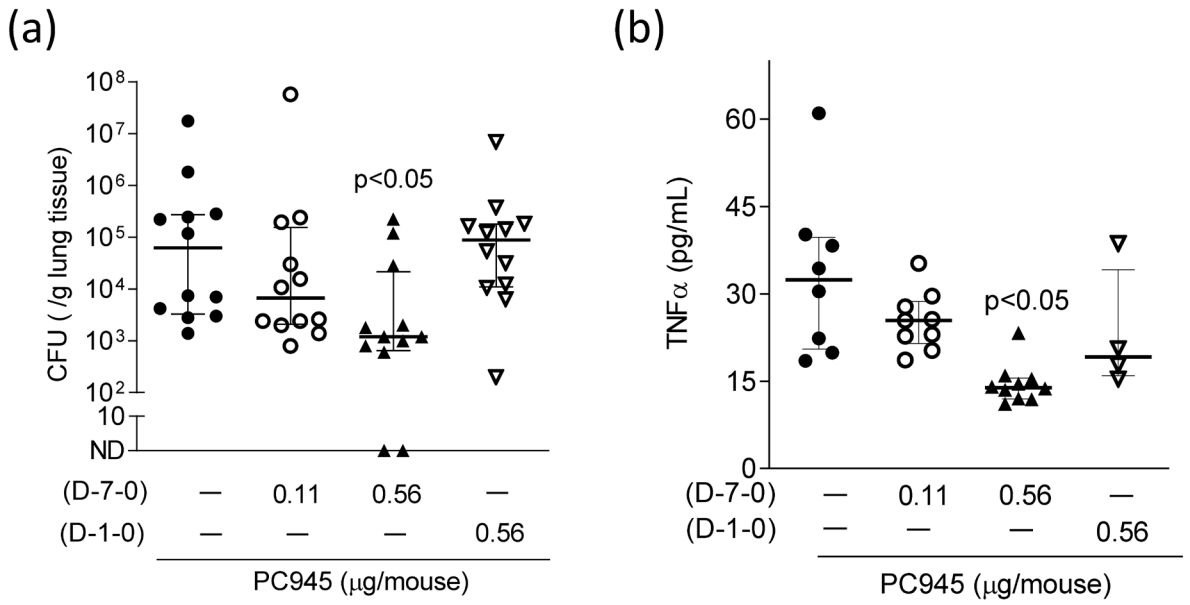

Figure 5: Antifungal activity of extended prophylaxis with PC945 against *C. albicans* in immunocompromised mice. Effects of 8 days extended prophylaxis with intranasal PC945 (0.11, 0.56 μg/mouse, once daily) was compared with that of 2 days prophylaxis treatment only on lung fungal load (CFU/g tissue) (a) and TNFα in serum (b). Each horizontal bar shows the median with interquartile range.

		PC945			**Voriconazole**		
	Number of isolates tested	**GM-MIC**	**MIC_{50}**	**MIC_{90}**	**GM-MIC**	**MIC_{50}**	**MIC_{90}**
Candida albicans	37	0.017	≤0.016	0.125	≤0.016	≤0.016	0.0625
Candida tropicalis	7	0.063	0.0312	>8	0.052	0.0312	2
Candida parapsillosis	17	0.017	0.0312	0.0312	≤0.016	≤0.016	≤.0.016
Candida glabrata	10	0.12	0.125	0.25	0.044	0.0625	0.125
Candida lucitaniae	11	≤0.016	≤0.016	0.0312	≤0.016	≤0.016	≤0.016
Candida guilliermondi	6	0.18	0.25	0.5	0.035	0.0312	0.0625
Total	88	0.027	0.0312	0.25	≤0.016	≤0.016	0.0625

All MICs were determined visually using the azole endpoint; GM: Geometric Mean; MIC_{50}: 50% percentile MIC, MIC_{90}: 90% percentile MIC, Assays were conducted with CLSI M38 guidelines, and the plate was read after 24h incubation.

Table 3: Susceptibility testing of *Candida* spp. to PC945 by CLSI.

to or less potent than voriconazole and more potent than posaconazole (Table 3, Figure 6a). All azoles used showed high MIC values for *C. tropicalis* FA1572 strain, which is known to be fluconazole resistant (Figure 6b). MIC values of voriconazole and posaconazole for the quality control strains (*C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019)] were within the expected MIC range (0.063-0.125 and 0.125-0.25 for *C. krusei*, 0.016 and 0.063-0.125 for *C. parapsilosis*, respectively).

In addition, activities against same *Candida* isolates tested above were also evaluated using the EUCAST method. The geometric mean MIC, MIC_{50} and MIC_{90} values of PC945 against all *Candida* spp. tested were 0.045, 0.016 and 1 μg/mL, respectively, >2-fold less potent than voriconazole (Table 4) as was observed using the CLSI method. Again, the MIC_{90} on *C. tropicalis* and *C. guilliermondii* for PC945 and other antifungals are relatively higher because of limited isolate number tested. There was a good correlation

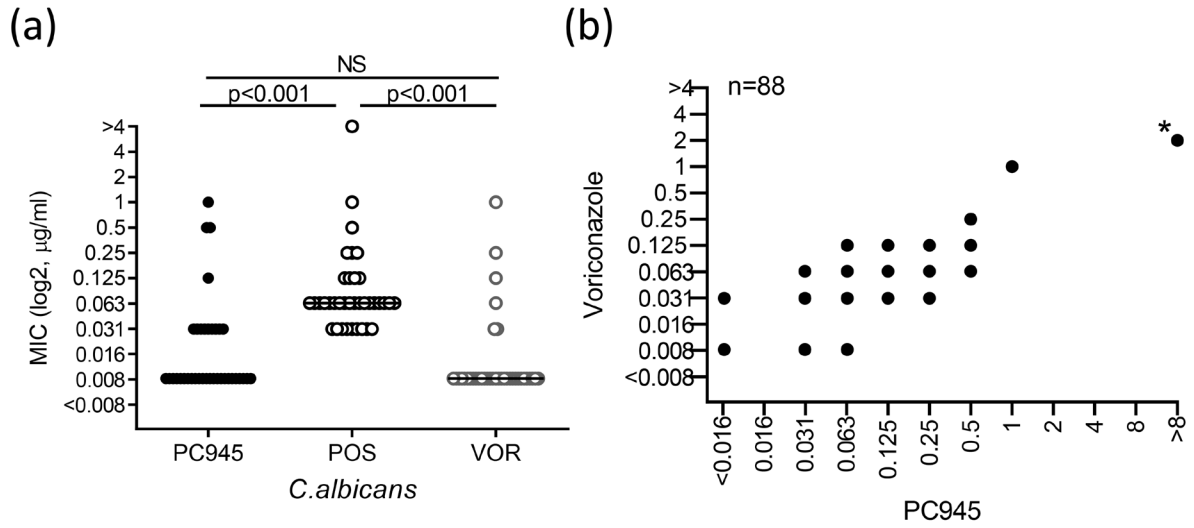

Figure 6: MIC distribution analyses: Individual MIC distribution versus *C. albicans* isolates **(a)** and the relationship of MIC distribution against all *Candida* species between PC945 and voriconazole **(b)** **C. tropicalis* FA1572 strain. POS: posaconazole; VOR: voriconazole.

		PC945			**Voriconazole**		
	Number of isolates tested	**GM-MIC**	**MIC_{50}**	**MIC_{90}**	**GM-MIC**	**MIC_{50}**	**MIC_{90}**
Candida albicans	37	0.024	0.0156	0.125	≤ 0.008	≤ 0.008	0.125
Candida tropicalis	7	0.076	0.0312	>4.0	0.051	0.0312	2.0
Candida parapsillosis	17	0.031	0.0312	0.625	≤ 0.008	≤ 0.008	0.0156
Candida glabrata	10	0.54	1.0	2.0	0.095	0.125	0.25
Candida lucitaniae	11	0.019	0.0156	0.0312	≤ 0.008	≤ 0.008	0.0156
Candida guilliermondi	6	0.18	0.25	4.0	0.040	0.125	0.125
Total	88	0.045	0.0156	1.0	0.012	≤ 0.008	0.125
All MICs were determined visually using the azole endpoint; GM: Geometric Mean, MIC_{50}: 50% percentile MIC, MIC_{90}: 90% percentile MIC, Assays were conducted with EUCAT E.DEF 7.3 guidelines, and the plate was read after 24h incubation.							

Table 4: Susceptibility testing of *Candida spp*. to PC945 by EUCAST.

between the values from the CLSI and EUCAST tests (Pearson r=0.84, p<0.0001). MIC values of voriconazole versus the quality control strains (*C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019)] were within the expected MIC range (0.25 and 0.016, respectively).

Discussion

PC945 has been found to be a potent antifungal agent, possessing activity against a broad range of *Candida* species, more potent than posaconazole, but comparable to or weaker than voriconazole in *in vitro* broth assays. *In vitro* anti-fungal activity of PC945 against the *C. albicans* (ATCC MYA4901 [529L]), used for *in vivo* analysis, was also slightly weaker than or similar to that of voriconazole. However, despite the weaker or similar *in vitro* potency, PC945 was 7~25-fold more potent than voriconazole in *C. albicans* infected mice *in vivo* when dosed intranasally once daily.

Although there is growing evidence that colonization of the lung by *C. albicans* impacts the pathogenesis of chronic respiratory diseases, experience with animal models to assess *in vivo* efficacy of antifungal agents is limited. Xu *et al.* reported a model where *C. albicans* was dosed intratracheally, showed acute lung injury and airway inflammation in both intact and immunosuppressed BALB/c mice, but the infection was only lethal in immunosuppressed mice [44]. In this report, we used C5 deficient A/J mice which were found to be very susceptible to *A. fumigatus* infection [37, 40], with histology revealing significant airway inflammation and moderate acute lung injury.

In this model, PC945 was dosed to animals, followed 24 h later by inoculation with *C. albicans*. To evaluate biomarkers, all surviving animals were sacrificed on Day 5, so survival was not monitored thereafter. 79% of vehicle treated *C. albicans* infected control mice were withdrawn from the study by Day 5. However, once-daily treatment with PC945 showed dose-dependent beneficial effects on survival post inoculation with a statistically significant effect at 14 μg/mouse (ED_{50}=6.4 μg/mouse). Voriconazole showed statistically significant beneficial effects on survival at 350 μg/mouse (ED_{50}=70 μg/mouse), suggesting PC945 is much more potent than voriconazole *in vivo*. For fungal burden analysis (the dose inducing 3 Log reduction), PC945 was found to be 6.8-fold more potent than voriconazole. Despite displaying similar antifungal activities to voriconazole in broth microdilution assays *in vitro*, these results clearly indicate that PC945 significantly outperforms voriconazole *in vivo*.

This superior profile probably arises from several factors, including pharmacokinetics, physicochemical properties and unique pharmacological characteristics. As reported earlier, PC945 has been demonstrated to have a longer duration of action in bronchial epithelial cells and hyphae than voriconazole [32]. Moreover, the molecule is retained within the lung, resulting in low systemic exposure after oral and face mask nebulization in a phase 1 study (NCT02715570) [36]. Although patients have been treated with voriconazole by nebulization successfully [27], it required frequent dosing at extremely high doses due to its short duration of action in the lung and rapid systemic exposure after nebulization [27,28]. Voriconazole's short pharmacodynamic duration of action in the lung relative to PC945 probably contributed to the relative antifungal activities seen here, where a once daily regimen was used. Voriconazole is also known to induce its own metabolism in mice, thus resulting in suboptimal exposures (AUC) in the lungs of mice compared with those in humans [45,46]. However, Andes showed potent effects of voriconazole on candidiasis in neutropenic mice [47], and in this manuscript, we applied voriconazole to the lung by an intranasal injection at much higher local concentrations without systemic metabolism (via liver). The PK after intranasal treatment of voriconazole was not reported and not evaluated in this manuscript, and the lung concentrations can be evaluated in future. In addition, unique physicochemical properties might contribute to the beneficial character of PC945. As shown in Table S3, PC945 is not Lipinski-compliant, with a high molecular weight, topological polar surface area (TPSA) and Log P value, so its physio-chemical properties are markedly different from those of voriconazole. PC945 also demonstrates lower aqueous solubility than voriconazole. As discussed above, PC945 has been optimized for a long residence time in airway epithelial cells and hyphae, but the molecular mechanism(s) leading to long lung residency and long lung duration of action have not been fully elucidated. Thus, all of the differences in *in vivo* effect could be attributed to a higher PC945 exposure (AUC) in the lungs and probably not due to PC945 being more potent than voriconazole a shown in MIC assay. Further studies are required to identify the mechanisms which produce PC945's superior *in vivo* performance.

In addition, we have demonstrated here that 8-day prophylactic treatment (using very low doses) produced much greater anti-*Candida* activity than prophylactic treatment limited to 2 days. Furthermore, the effects of 8-day prophylactic treatment were maintained when treatment ceased just before *Candida albicans* inoculation on day 0 (Figure 5). This is powerful pharmacodynamic evidence that the effects of PC945 accumulate on daily dosing in mice and are maintained when dosing ceases. In fact, both our phase 1 study with healthy volunteers and rat studies demonstrated that PC945 accumulated in lung after repeat daily dosing (NCT02715570) [36].

Interestingly, Tolman and colleagues have demonstrated that prophylaxis with aerosolized aqueous intravenous formulation of voriconazole significantly improved survival and limited the extent of invasive disease with *Aspergillus fumigatus*, as assessed by histopathology, in an invasive pulmonary murine model [26]. Recently, Baistrocchi and colleagues reported that posaconazole accumulated in granulocyte type cells leading to enhanced anti-fungal effects (by exposure of cellular posaconazole during phagocytosis) [48]. We have also demonstrated PC945 accumulation into alveolar cells in mice, including macrophages, after intranasal treatment [49] and showed that PC945 changes the cell integrity of *A. fumigatus* to accelerate elimination by macrophages/neutrophils [50]. It is possible that loading of PC945 into granulocyte/macrophages might enhance the compound's intrinsic anti-fungal activity against *C. albicans*.

Against 88 *Candida* isolates, there was a good correlation between the MIC values of PC945 and those of voriconazole. One isolate, *C. tropicalis* FA1572, which is known to be fluconazole resistant with a mutation of CYP51 at Y132 [51,52], was found to be resistant to both treatments. Between species, the activities of PC945 against *C. albicans*, *C. tropicalis, C. parapsilosis* and *C. lucitaniae* were more potent than those against *C. glabrata* and *C. guilliermondi*. As previously reported, PC945 also inhibited the growth of *C. auris* (n=72) [34] and *C. krusei* (n=1) [32]. The effects of PC945, determined by the CLSI method, were confirmed using the EUCAST method. MICs against *Candid*a species obtained by the CLSI and EUCAST methods are reported to show a good correlation for fluconazole and voriconazole in general, whereas MICs obtained by the EUCAST method are typically lower for posaconazole [53] than those obtained using CLSI. For PC945, there was a good correlation between results obtained using the two methods.

This study had several limitations. Firstly, this model is unlike natural chronic respiratory colonization seen in asthma or COPD, more closely resembling an immunocompromised setting (invasive form). We did not evaluate clinically relevant markers, such as lung function or symptoms, as readouts. Therefore, it may be inappropriate to extrapolate the results of this study directly to chronic respiratory disease with persistent *Candida* colonization in the clinic. However, we believe that this model is still useful, allowing assessment of the anti-fungal, pharmacodynamic properties of PC945. Secondly, the most of reports of *Candida* infection in the respiratory tract are still on a case-reportable basis and no placebo-controlled intervention study on *Candida* infection was reported. Thirdly, there was a lack of pharmacokinetic measurements of PC945 in mice used in the current experiments to help understand the superior anti-fungal effects of PC945 versus voriconazole. Preliminary data suggested accumulation of PC945 in the lung after repeat intranasal treatment in *A. fumigatus* infected mice [49]. As we used mice in the same condition (immunocompromised, temporally neutropenic) as this previous study, we expect similar pharmacokinetics in the current experiment. However further PK-PD analysis in this *Candida* infection model should be conducted to confirm this in the future. Finally, we showed CFU data as per gram of lung tissue. There might be many confounders that influence organ weight, such as condition of mice (oedema etc.), variable volume of blood remained etc. However, we were not able to normalize to dried tissue weight as we had to get yeast from fresh lung tissue. As the difference of CFU between groups is >3 log, those factors are only likely to have a limited impact on the results.

Thus, we showed superior *in vivo* performance of PC945 versus voriconazole on fungal load and survival rate after intranasal treatment against *C. albicans* lung infection, replicating our findings using *Aspergillus fumigatus* infection [40]. Further data suggested that the antifungal effects in the lung of PC945 accumulated on repeat dosing and that these effects were persistent. Thus, PC945 has the potential to be a novel inhaled therapy for the prevention or treatment of *C. albicans* lung infection in humans. PC945 is in clinical development [54], and these *in vivo* data indicate that further clinical evaluation is warranted.

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Author Contributions

Conceptualization, K.I., Y.K. and P.S.; Methodology, Y.N., K.I., G.K. and Y.K.; Validation, K.I. and Y.K.; Formal analysis, K.I. and Y.K.; Investigation, Y.N., G.K. and L.D.; Data curation, K.I., Y.N., G.K. and Y.K..; Writing—original draft preparation, K.I. and Y.K.; Writing—review and editing, Y.N., G.K., K.L., L.D. and P.S.; Supervision, K.I. and Y.K.; Project administration, K.I. and Y.K.; Funding acquisition, P.S. and K. Y. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

P. Strong and K. Ito are employees and (co) founders of Pulmocide Ltd., and have stock option. K. Lucas and L. Daly are employees of Pulmocide Ltd. Y. Kizawa received

funding from Pulmocide for *in vivo* experiments. Other authors declare no conflict of interest.

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