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Title

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

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ABSTRACT

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

Antimicrobial Agents and Chemotherapy

INTRODUCTION

The current management of the three major forms of aspergillosis: invasive aspergillosis (IA), chronic pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis (ABPA) (1-4), involves prophylactic or therapeutic administration of triazoles and, infrequently, surgical intervention (5). Existing antifungal medicines are predominantly dosed either orally or systemically. These frequently exploited 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. This is especially so for the liver, which is a site of triazole toxicity: up to 15% of patients treated with voriconazole experience raised transaminase levels (6, 7). Exposure of the liver also results in significant drug interactions arising from triazole inhibition of hepatic P450 enzymes (8, 9).

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

70 difluorophenyl)-5-(1*H*-1,2,4-triazol-1-ylmethyl)oxolan-3-yl]methoxy}-3-

71 methylphenyl)piperazin-1-yl]-*N*-(4-fluorophenyl)benzamide (Structure in Fig. 1A); a novel,

72 triazole, antifungal agent designed specifically for inhaled administration (10).

73

MATERIALS AND METHODS

Antifungal agents. 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. PC945 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.

***A. fumigatus* CYP51 binding assay and enzyme inhibitory activity.** *A. fumigatus* CYP51 binding properties were determined as described by Warrilow *et al.* (11). Test agents were titrated against 4 µM recombinant *A. fumigatus* 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 (12).

A CYP51 reconstitution assay system was used to determine 50% inhibitory (IC_{50}) concentrations (13). 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^{-1} 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 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 PC945 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 experimentation.

Non-saponifiable lipids were extracted as previously reported (14) 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 (15). Sterol composition was calculated using peak areas from the gas chromatograms and the mass fragmentation pattern 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 PC945) were determined.

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

Fluorescent imaging of *A. fumigatus* infected cells. Human alveolar epithelial cells (A549) were seeded onto collagen-coated coverslips and incubated at 37°C and 5% CO_2 for 24 h. Cells were incubated in the presence of test agents for 2 h, after which the media was replaced and the coverslips were incubated at 37°C, and 5% CO_2 for 24 h. CellTracker red CMTPX dye (ThermoFisher, C34552) was added to cell media for 30 minutes, wells were washed with PBS, and GFP-*A. fumigatus* conidia, [a kind gift from Professor William Hope, University of Liverpool], were added to wells at a final concentration of 1 x 10^3 spores ml^{-1}. After 24 h incubation at 35°C and 5% CO_2, coverslips were washed and affixed to slides using Fluoroshield™ with DAPI (Sigma, F6057).

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

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

***In vitro* antifungal activity against other fungal species.** For the measurement of activity against *Cryptococcus 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 (17). Antifungal potency against *Aspergillus flavus, Aspergillus niger* and *Aspergillus terreus*, was determined as set out in EUCAST definitive document EDef 9.2 and assay plates were incubated at 37°C for 48 h (18). 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 (19) or M27-A2 (20), www.eurofinspanlabs.com).

***In vitro* determination of persistence of action on *A. fumigatus* hyphae.** The persistence of action of test agents was calculated in *A. fumigatus* hyphae (NCPF2010). Conidia were diluted in growth media (RPMI-1640, 2 mM L-glutamine, 2 % glucose, 0.165 M MOPS, pH 7.0) and 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 λex/λem 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).

***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). 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 1×10^3/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).

***In vivo* antifungal activity against *A. fumigatus* 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 ± 1ºC) 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 (21). 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 (22). To avoid bacterial infection, drinking water was supplemented with tetracycline hydrochloride (Sigma T7660; 1 µg/ml) and ciprofloxacin (Fluka 17850; 64 µg/ml). *A. fumigatus* (ATCC13073 [strain: NIH 5233], purchased from the American Type Culture Collection, Manassas, VA, USA) was grown on malt agar (Nissui Pharmaceutical, Tokyo, Japan) plates for 6–7 days at room temperature (24 ± 1ºC). Conidia were aseptically dislodged from the agar plates and suspended in sterile distilled water with 0.05% Tween 80 and 0.1% agar. On the day of infection, conidial counts were assessed by haemocytometer and the inoculum was adjusted to obtain a concentration of 1.67×10^8/ml in physiological saline. On day 0, 30 µl of the conidia suspension was administered intranasally.

Test agents, suspended in physiological saline, were administered daily intranasally (35 µl) on days 1 to 6 and the survival of animals was recorded for 7 days. The volume inserted intranasally is reported to achieve almost 60% deposition into the lung (23). Deaths and the body weights of surviving animals were monitored daily. 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. It was observed that *A. fumigatus* infection induced a "rolling" behaviour, which was monitored and recorded (24). Bronchoalveolar lavage fluid (BALF) was collected on day 7 post infection or on the day that the mouse dropped out of the study. The *Aspergillus* GM concentration in BALF 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. 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). Survival analysis was performed by Kaplan-Meier plots followed by the log rank (Mantel-Cox) tests using the PRISM 6® software program (GraphPad Software Inc., San Diego, CA, USA). For comparison between groups either the ordinary one-way ANOVA with Tukey's *post hoc* comparison, or the Kruskal-Wallis ANOVA with Dunn's *post hoc* comparison was performed. Statistical significance was defined as $P<0.05$.

RESULTS

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

Inhibitory activity against *A. fumigatus* CYP51 enzyme. The inhibitory activity of PC945 and posaconazole 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 *E. coli* expression clones. Both PC945 and posaconazole were strong tight-binding inhibitors of CYP51A and CYP51B *in vitro* activity (Table 1; Fig. 1C), suggesting $K_{i.app}$ values of below 1 nM for both compounds (25). Moreover, PC945 was equally effective as posaconazole, and both agents appear to share the same mode of action; by directly coordinating as the sixth axial ligand of the CYP51 heme iron. No allosterism was observed during the inhibition of AfCYP51B activity by PC945.

Cell based *A. fumigatus* sterol composition and CYP51 assay. Analysis of sterol composition was performed by GC-MS. Treatment with increasing concentrations of either posaconazole or PC945 from 0 to 1 μg ml^{-1} 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 2, Fig. 1D).

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

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

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

***In vitro* antifungal activity against clinically isolated *A. fumigatus*.** Test agents were evaluated in 50 clinical isolates obtained from the Saint Louis Hospital (Paris, France) and 46 clinical isolates obtained from the NW Mycology Centre in the UK. PC945 was found to be 2.5 fold more potent than voriconazole but comparable to posaconazole based on the geometric mean (Table 4). Among the clinical isolates from the NW Mycology Centre, 13 of the 46 strains were found to be posaconazole resistant, 7 of 13 posaconazole resistant isolates were confirmed as itraconazole resistant, and two of them were pan-azole resistant including voriconazole, based on the EUCAST ECOFF. In five of the posaconazole resistant isolates, PC945 did not inhibit growth completely at concentrations up to 8 µg/ml. During this assay, the quality control strain *A. fumigatus* ATCC204305 was used for validation. In this strain 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.** Visual assessment of the growth of four itraconazole susceptible *A. fumigatus* strains demonstrated that PC945 was the most potent compound tested with an MIC value of 0.031 µg/ml; whilst voriconazole and posaconazole were less effective (Table 5). Thus the superiority of PC945 to voriconazole on *A. fumigatus* growth inhibition was confirmed by the CLSI method as well as the EUCAST microdilution method.

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

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

351 growth, arising from continuous contact of the test compounds with *A. fumigatus* hyphae for 16
352 h, was measured and compared with that obtained after contact with drug for only 20 minutes,
353 followed by washout and incubation for the same period. As seen in Table 7 and Fig. 2B, the
354 antifungal potency of voriconazole and posaconazole diminished markedly, after short contact
355 and washout, by a factor of > 93-fold and 4.9-fold, respectively. In contrast, PC945 showed no
356 change in its antifungal activity between wash and non-wash in this experimental paradigm
357 (Table 7). It was also observed that PC945 (IC_{50}: 0.00010 μg/ml) was 110 and 4.5-fold more
358 potent, than either voriconazole or posaconazole at inhibiting hyphal *A. fumigatus* growth.
359 In the second system, the persistence of action of PC945 was quantified using GM
360 production in supernatant as an index of fungal growth. BEAS2B cells were infected with *A.*
361 *fumigatus*, and the effects of a 24 h washout period prior to infection were examined. A one hour
362 contact time with PC945 followed by 24 h washout resulted in an approximate 11-fold loss of
363 potency against *A. fumigatus*, compared with the control in which there was no washout.
364 Although posaconazole showed a similar or slightly greater loss of potency on washout, it was
365 notable that voriconazole was ineffective after a 24 h washout. These data imply that only a short
366 contact period of bronchial epithelial cells with PC945 would be required for the agent to exhibit
367 a long duration of therapeutic action (Table 7, Fig. 2C).
368
369 ***In vivo* antifungal activity.** The potential of intranasally administered PC945 as a daily
370 therapeutic treatment for pulmonary *Aspergillus* infection was investigated using
371 immunocompromised, temporarily neutropenic mice. In this model, 81% of control mice (13/16)
372 were dead or had dropped out by day 6 post infection and only 19% of mice survived (Table 8,
373 Fig. 2D). However, it was observed that 50, 63 and 63% of mice dosed with PC945 (0.56, 2.8
374 and 14 μg/mouse: intranasal application of 0.016, 0.08 and 0.4 mg/ml suspension) survived

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

DISCUSSION

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

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

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

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

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

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

The proposed mechanism of action of PC945 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 (40, 41). PC945 produced type II difference spectra when titrated against purified *A. fumigatus* CYP51A and CYP51B enzymes, and bound with a similar affinity to CYP51A as posaconazole, but yielded a sigmoid binding saturation curve against CYP51B. This latter binding characteristic of PC945 was not reflected in the compound's inhibition of CYP51B activity, suggesting a difference in the enzyme's conformation in solution and that adopted when incorporated in cell membranes. Furthermore, the strong inhibition of CYP51A activity observed with both PC945 and posaconazole, indicative of tight-binding inhibitors (IC_{50} value approximately half that of the enzyme concentration present) was tighter than predicted by the calculated K_d values from ligand binding studies using recombinant CYP51A, suggesting the conformation of purified CYP51A in solution differs to that in cell membranes. In the sterol composition assay, treatment with

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

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. In order to explore this parameter the persistence of action of PC945 in a number of *in vitro* systems was studied. In *A. fumigatus* hyphae the IC_{50} value measured for PC945 following a 20 minute contact period and washout for 16 h was almost unchanged 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 10-fold loss of potency against *A. fumigatus* compared with control in which there was no washout period. This property of cellular persistence, in the absence of the pathogen, may be a particularly valuable property by enhancing the potential use of PC945 in prophylaxis.

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

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

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

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

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

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FIGURE LEGEND

FIGURE 1 Efficacy of PC945 as an inhibitor of *A. fumigatus* sterol 14α-demethylase (CYP51). (a) Structure of PC945, (b) Type II azole binding spectra for *A. fumigatus* CYP51A and CYP51B, each experiment was performed 4-6 times although only one replicate is shown (c) Azole IC_{50} determinations of posaconazole (●) and PC945 (○), mean relative velocity values are shown with standard deviations, (d) Sterol composition of *A. fumigatus* treated with PC945, the relative levels of lanosterol and eburicol.

FIGURE 2 Antifungal activity of PC945 against *A. fumigatus in vitro* and *in vivo*. (a) A549 treated with PC945 or posaconazole and infected with GFP-*A. fumigatus*: green = GFP-*A. fumigatus*; Blue = DAPI-stained nucleus; Red = Celltracker-stained cytoplasm, (b) Persistence of action of PC945 and voriconazole on *A. fumigatus* hyphae, (c) Persistence of action of PC945 and voriconazole on human bronchial cell lines (Beas2B) infected with *A. fumigatus*, (d, e) Effect of once daily intranasal treatment of PC945 (0.56, 2.8, 14 µg/mouse) and posaconazole (2.8, 14, 70 µg/mouse) on survival in *A. fumigatus* infected immunocompromised mice (N=8~16), (f) Galactomannan in BALF, each horizontal bar was presented as mean ± SEM from 8 mice per group. $^{\#}$ Dead before day 7. *** Significant difference from no infection at $P<0.001$. ††† Significant difference from infection control at $P<0.001$.

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

Test Agent	AfCYP51A		AfCYP51B		
	K_d (μM)[a]	IC_{50} (μM)[b]	K_d1 (μM)[a]	K_d2 (μM)[a]	IC_{50} (μM)[b]
PC945	0.50	0.23	19298	0.32	0.22
Posaconazole	0.96	0.16	0.012	n/a	0.17

685 [a] K_d determinations used 4 μM purified AfCYP51A and 4 μM AfCYP51B proteins.

686 [b] IC_{50} determinations used 0.5 μM AfCYP51A and 0.5 μM AfCYP51B recovered in the

687 membrane fraction from the *E. coli* expression clones.

688 n/a = not applicable

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

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

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

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Antimicrobial Agents and Chemotherapy

720 **TABLE 3** Antifungal effects of PC945 and known antifungal agents in azole susceptible and azole resistant strains of *A. fumigatus* [a]

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

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

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Antimicrobial Agents and Chemotherapy

731 **TABLE 4** *In vitro* activities of PC945, posaconazole and voriconazole against 96 clinically
732 isolated *A. fumigatus* strains [a]

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

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

735 [b] Geo-mean = geometric mean.

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

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751 **TABLE 5** Antifungal effect of PC945 and known antifungal agents on 4 itraconazole susceptible

752 *A. fumigatus* strains (CLSI methodology)

	MIC (µg/ml)[a]		MIC_{50} (µg/ml)[b]	
Test Agent	**Median**	**Interquartile**	**Median**	**Interquartile**
PC945	0.031*	0.020–0.031	0.011**	0.0083–0.024
Voriconazole	0.5	0.5–0.5	0.14	0.082–0.15
Posaconazole	0.047	0.031–6.0	0.015	0.0095–0.016

753 [a] MIC determined visually.

754 [b] MIC_{50} determined using optical density measurements.

755 * $P < 0.05$, ** $P < 0.01$; PC945 versus the results for voriconazole (Kruskal-Wallis one-way

756 ANOVA with Dunn's test).

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

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

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

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

[b] MIC given is MIC_{50}.

AR = azole resistant, fluconazole and voriconazole.

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

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

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

793 Dunn's test).

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TABLE 8 *In vivo* activities of PC945 and posaconazole

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

n = number; N = total.