Effects of intranasally dosed posaconazole on fungal load and biomarkers in *Aspergillus fumigatus* infected immunocompromised mice

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Summary
Although anti-fungal triazoles are dosed orally or systemically for *Aspergillus fumigatus* infection, systemic adverse events and limited exposure of the lung cavity would make a topical treatment for the lung an attractive option. In this study, we examined the effects of intranasally dosed posaconazole on survival rates and biomarkers in *A. fumigatus* (itraconazole susceptible: ATCC13073 [Af]; or resistant: NCPF7100 [AfR]) infected, temporarily neutropenic A/J mice. Once daily treatment produced a dose-dependent improvement of survival of Af- infected mice (ED50: 0.019 mg/mouse [approx. 0.755 mg/kg, in]), similar to its potency (ED 50: 0.775 mg/kg, po) after once daily oral dosing. For AfR infection, either intranasal or oral posaconazole was largely ineffective on survival, although the highest dose of intranasal treatment (0.35 mg/mouse) achieved 75% survival rate. Early intervention (treated on days 0, 1, 2 and 3 postinfection) and late intervention (treated on days 1, 2 and 3) with intranasal posaconazole (0.014-0.35 mg/mouse) demonstrated potent inhibition of lung fungal load and galactomannan levels in both bronchoalveolar lavage fluid (BALF) and serum as well as inflammatory cells, IFN-γ, IL-17 and malondialdehyde (MDA) in BALF. Thus, posaconazole when dosed intranasally once daily showed an improvement of survival equivalent to or better than oral treatment, and produced potent inhibition of fungal load and biomarkers.

**KEYWORDS**
*Aspergillus fumigatus*, galactomannan, intranasal treatment, itraconazole resistant, posaconazole

1 | INTRODUCTION
Posaconazole is a second-generation triazole anti-fungal agent with a broad spectrum, and has been approved for treatment of pulmonary aspergillosis. However, oral treatment of posaconazole occasionally shows systemic side effects including diarrhoea, nausea, QTc prolongation and rarely torsade de points. In addition, *Aspergillus* colonisation following infection can occur in preexisting lung cavities caused by tuberculosis or sarcoidosis or on airway surfaces where the fungus firstly deposits and grows, and it is difficult to deliver and maintain appropriate levels of the treated drug after oral treatment at these sites. Thus, there are several advantages of topical treatment over oral/systemic treatment which favourably alter the risk-benefit ratio of treatment.

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Amphotericin B, a polyene anti-fungal agent, is the only anti-fungal agent dosed via the inhaled route.® The agent has poor oral absorption and its use as an intravenous infusion is complicated by an adverse effect on the kidney.® Inhaled amphotericin B is effective in treatment and its use as an intravenous infusion is complicated by an adverse effect on the kidney.® Voriconazole, a triazole with potent anti-fungal activity against Aspergillus species, is available as oral administration or intravenous infusion. Recently voriconazole was also dosed via the pulmonary route, and showed good efficacy,9 avoiding drug-drug interactions® and systemic side effects®. The main drawbacks of inhaled amphotericin B and voriconazole include poor adherence to treatment because frequent administration is required.

Itraconazole is another azole used to treat Aspergillus infection. McConville et al.® demonstrated that a single dose of nebulised itraconazole maintained high concentrations in lung in mice for more than 24 hours and it has also been demonstrated in rats.® Furthermore the survival rates of the group of mice receiving nebulised itraconazole were significantly higher than the group of mice receiving oral itraconazole administration.® Posaconazole, the most potent triazole, has longer tissue retention than itraconazole or voriconazole,® but topical treatment of posaconazole, as a potential option for the treatment, has not been tested in vivo.

In this study, therefore, we examined the effects of intranasally dosed posaconazole on biomarkers and survival rates vs both itraconazole-susceptible (ATCC13073: Af) and -resistant (NCPF7100: AfR) Aspergillus fumigatus-infected immunocompromised mice, and in comparison with those obtained after oral treatment.

2 | MATERIALS AND METHODS

2.1 | Materials

The materials used were obtained from the following sources: Tetracycline hydrochloride and ciprofloxacin (Sigma-Aldrich Co, LLC, St. Louis, MO, USA); fluorescein isothiocyanate (FITC)-conjugated anti-macrophage (MOMA2) antibody and FITC-anti-neutrophils (7/4) antibody (Acris Antibodies GmbH, Herford, Germany). Posaconazole, voriconazole and itraconazole were sourced from Apichem Chemical Technology Co., Ltd. (Zhejiang, China), Tokyo Chemical Industry UK Ltd. (Oxford, UK) and Wako Pure Chemical Industries Ltd (Tokyo, Japan), respectively.

2.2 | Animals

Specific pathogen-free A/J mice (male, 5 weeks old) were purchased from Sankyo Labo Service Co. Ltd. (Tokyo, Japan) and adapted for 1 week in a temperature (24 ± 1°C) and humidity (55% ± 5%) controlled room with a 12 hours day-night cycle. The mice were reared on a standard diet and tap water ad libitum.

2.3 | Aspergillus fumigatus strains

The itraconazole-susceptible strain, ATCC13073 (Af; NIH 5233 strain), and itraconazole-resistant strain, NCPF7100 (AfR; AF90, M220V mutation), were purchased from American Type Culture Collection (Manassas, VA, USA) and Culture Collections, Public Health England (Salisbury, UK), respectively.

2.4 | MIC determinations

Anti-fungal susceptibility testing was performed by the EUCAST method (EUCAST definitive documents EDef 9.3 [2015]).® Briefly, stock solutions of test agents were prepared in neat DMSO, and were diluted 200-fold the desired concentrations with DMSO. A final DMSO was applied to fungus growth media to make sure the final concentration of DMSO was 0.5% (v/v) throughout all the plates evaluated. Flat-bottomed 96-well polystyrene plates (Corning 3370; Corning Inc., Corning, NY, USA) were inoculated at 35 ± 1°C for 24-48 hours as appropriate. The MIC endpoints were determined visually according to EUCAST guidelines.

2.5 | Animal model and biomarker analysis

2.5.1 | Aspergillus fumigatus infection and drug treatment

Both A. fumigatus strains were grown on Malt agar (Nissui Pharmaceutical, Tokyo, Japan) and spores were aseptically dislodged from the agar plates and suspended in physiological saline. To induct immunosuppression and neutropenia, A/J mice were dosed with hydrocortisone (Sigma H4881; 125 mg/kg, sc) on days 3, 2 and 1 before infection, and with cyclophosphamide (Sigma C0768; 250 mg/kg, ip) 2 days before infection as previously reported.® On day 0, animals were infected with the spore suspension (30 μL, intranasally 15 μL into each nostril) under anaesthesia with 3% isoflurane.

To prepare the aqueous suspension of posaconazole, solid material of posaconazole (10 mg) was added to 1 mL physiological saline, and it was sonicated for 1 minute (20 second×3 times) using sonicator (US-2R; AS ONE, Osaka, Japan). The stock solutions were diluted with physiological saline. Based on previous report showing that posaconazole concentration in epithelial cell lining fluid was 78.78 AUC mg h L−1 after dose of 32 mg/kg, po in mouse, the middle range of dose was set up as 0.014 μg/mouse.®

2.5.2 | Survival study

Animal body weights were monitored daily and if it fell by >20% compared to that on day 1 (when posaconazole treatment started), animals were defined as dropout and were sacrificed using a high dose of pentobarbital. Posaconazole was administered either intranasally (35 μL of a suspension in physiological saline, approximately 17.5 μL into each nostril) under anaesthesia with 3% isoflurane or orally (5 mL/kg of a suspension in PEG400 in physiological saline) once daily for 7 days from 30 minutes before infection or from a day after conidia inoculation. Particularly, the volume used for intranasal treatment (35 μL) is reported to achieve 60% deposition of lung.®
2.5.3 Sample collection for biomarker analysis

Posaconazole or vehicle was administered either intranasally under anaesthesia with 3% isoflurane or orally once daily, on day 0 (30 minutes before conidia infection), 1, 2 and 3 (called early intervention) or day 1, 2 and 3 (late therapeutic intervention) in itraconazole-susceptible A. fumigatus (Af)-infected mice. The animals were terminally anesthetised 6 hours after the last dose of drug administered on day 3. Bronchoalveolar lavage fluid (BALF) was collected through cannulated tracheas using physiological saline as we previously reported,22 blood was then collected via cardiac puncture and finally lung tissue was removed for homogenate preparation. The number of alveolar cells in BALF was determined by haemocytometer and the numbers of alveolar macrophages and neutrophils were determined by FACS analysis (EPICS ALTRA II, Beckman Coulter, Inc., Fullerton, CA, USA) using anti-mouse MOMA2-FITC (macrophage) or anti-mouse 7/4 (neutrophil) as previously reported.23

2.5.4 Cytokine and malondialdehyde measurement

Measurement of IFN-γ, IL-17 and CXCL-1 in BALF was performed using Quantikine® mouse ELISA kit (R&D systems, Inc., Minneapolis, MN, USA). Malondialdehyde (MDA) (Malondialdehyde) analysis was performed using OxiSelect® TBARS Assay Kit (Cell Biolabs Inc, San Diego, CA, USA).

2.5.5 Galactomannan determination

Galactomannan (GM) in BALF and serum was determination using Platelia Aspergillus EIA kits (Bio-Rad Laboratories, Redmond, WA, USA). BALF and serum samples were mixed with sample treatment solution provided in the kit, then centrifuged and the both supernants were diluted 20- or 10-fold with PBS, respectively. Cut-off index (COI) was calculated by the formula: Cut-off index=(OD in sample)/ (OD in cut-off control provided as part of the kit).

2.5.6 Aspergillus fumigatus load determination (CFU)

One hundred milligram of lung tissue from left lobe was aseptically removed and homogenised in 0.2 mL of 0.1% agar in sterile distilled water. Serially diluted lung homogenates were plated on Malt agar plates (50 μL/plate), and incubated at 35 ± 1°C for 72-96 hours. The colonies of A. fumigatus on each plate were counted and the fungal titre presented here as CFUs per gram of lung tissue.

2.5.7 Whole lung histological analysis

Whole lungs were fully inflated by intratracheal perfusion with 10% formalin neutral buffer solution. Routine histological techniques were used to prepare paraffin-embed tissue, and 4-μm sections of whole lung were stained with haematoxylin and eosin, and Grocott’s methenamine silver (GMS) stain kit (Silver Stain Kit, Sigma HT100A).

2.6 Aspergillus fumigatus DNA analysis

DNA was extracted from either infected lungs or A. fumigatus with the Isoplant (Nippon Gene) according to the manufacture’s instruction. DNA amplification was performed with Premix Ex Taq™ (Takara Bio Inc., Kusatsu, Japan) in the 96-well optical reaction plate. Aspergillus fumigatus 18S rRNA gene fragments were amplified with the primer pair; 5′-GGCCCTTAA ATAGCCCGGT-3′ (SEQ ID No. 1) and 5′-TGAGGCCGATAGTCCCCCTA-3′ (SEQ ID No. 2), and hybridisation probe; 5′-FAM-AGCCAGGCGCCGCAAATG-TAMRA-3′ (SEQ ID No. 3)24 as previously reported.25 The amounts of A. fumigatus DNA in 50 ng of mice lung DNA were evaluated from the standard curve with 0.05-50 000 pg of DNA from A. fumigatus.

2.7 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. The ED_{50} values of posaconazole were determined from the dose-response curves, the survival rates and the ID_{50} values of posaconazole were determined from the dose inhibitory response curves of each marker and cytokine production using the PRISM 6® software program (GraphPad Software Inc., San Diego, CA, USA). Multiple comparison was performed by ANOVA followed by Dunnett’s multiple comparison test performed using the PRISM 6®. The comparison between two groups was performed by unpaired t-test with Welch’s correction or Mann-Whitney test. Statistical significance was defined as P < .05.

3 RESULTS

3.1 MICs for ATCC13073 (Af) and NCPF7100 (Afr)

Af strain was highly susceptible to posaconazole, which was eightfold more potent than voriconazole. In contrast, Afr strain was 17 times less susceptible to posaconazole, and >64-fold less susceptible to itraconazole, although voriconazole showed similar MIC values in both strains tested. Thus, Afr is resistant to posaconazole and itraconazole as previously reported (Table 1).26

3.2 Effects of intranasal or oral posaconazole on survival rate of itraconazole-susceptible and -resistant Aspergillus fumigatus-infected mice

Sixty-two per cent of A. fumigatus (Af) infected mice died or dropped out up to 8 days after infection. Posaconazole dosed intranasally once

| TABLE 1 Susceptibility testing of azoles on growth of Aspergillus fumigatus (ATCC13073) strain by EUCAST |
|---------------------------------------------------------------|
| **MIC: mg/L** | **ATCC13073** | **NCPF7100** |
| Posaconazole | 0.060 | 1 |
| Voriconazole | 0.500 | 1 |
| Itraconazole | 0.125 | >8 |
daily (0.0028, 0.014, 0.07 and 0.35 mg/mouse: approximately 0.11, 0.56, 2.8 and 14 mg/kg, in) showed dose-dependent improvement of the survival rate, with 100% surviving at the highest dose tested, whereas the rate was 38% in vehicle-treated control group (Figure 1A). The ED$_{50}$ calculated based on survival on Day 8 was 0.019 mg/mouse, po (0.755 mg/kg, in). Posaconazole given orally once daily also improved survival at 1 mg/kg, po (Figure S1) and the ED$_{50}$ calculated based on survival on Day 8 was 0.775 mg/kg, po. Thus, the ED$_{50}$ value of intranasal treatment was similar to that of oral treatment.

*Aspergillus fumigatus* (AFR) infection showed also poor survival rate in mice, which demonstrated similar kinetics with Af infection (Figure 1B). Up to 5 mg/kg, oral posaconazole did not show any improvement in the survival rate (Figure S1B). However, intranasal posaconazole achieved 75% survival rate at 0.35 mg/mouse (14 mg/kg, in) where vehicle-treated animals showed only 13% survival (Figure 1B).

We did not observe any adverse events associated with intranasal treatment of posaconazole in this dosing regimen.

### 3.3 | Kinetics of biomarkers in *Aspergillus fumigatus* (Af) infected temporarily neutropenic A/J mice

CFU was increased in *Aspergillus* infected lung from day 3 and peaked at between day 4 and day 6 (Figure 2A). The levels of GM determined as cut-off index (COI) in both BALF and serum also significantly increased after *A. fumigatus* infection and peaked between day 3 and day 6 (Figure 2B and C). The GM level went down to basal levels on day 8, although many of infected mice were dead or dropped out by day 8. Remarkable induction of *Aspergillus* 18S DNA in lung tissue was also observed on day 4 and day 6 (Figure 2D).

As well as the level of GM and CFU, inflammatory and other markers were elevated after *Aspergillus* infection. As seen in Figure 2E and F, alveolar macrophages and neutrophils in BALF were slightly increased by *A. fumigatus* infection. An oxidative stress marker, MDA (TBARS reactive molecule), showed induction just after infection, and then gradually increased between days 3 and 6 in BALF (Figure 2G). IFN-γ and IL-17 in BALF seemed to increase in a time-dependent manner up to day 8 (Figure 2H and I).

### 3.4 | Effects of intranasal posaconazole on fungal load and fungal biomarkers in *Aspergillus fumigatus* (Af) infected mice

Early intervention by intranasal posaconazole produced potent, dose-dependent inhibition of GM in BALF and serum (Figure 3A and B) with ID$_{50}$ values of 0.016 mg/mouse and 0.018 mg/mouse (approximately 0.63 mg/kg, in and 0.73 mg/kg, in) respectively. CFU in the lung was also reduced by early intervention with intranasal posaconazole (Figure 3C) and the ID$_{50}$ value was 0.013 mg/mouse (approx. 0.51 mg/kg, in), which was similar to those for BALF and serum GM. In addition, late intervention treatment of intranasal posaconazole also produced dose-dependent inhibition of GM in BALF and serum (Figure 3A and B) with ID$_{50}$ values of 0.021 mg/mouse (approx. 0.83 mg/kg, in) for both. CFU in the lung was also reduced by late intervention of intranasal posaconazole (Figure 3C) and the ID$_{50}$ value was 0.014 mg/mouse (approx. 0.55 mg/kg, in), which was similar to those for BALF and serum GM. These ID$_{50}$ values for BALF GM, serum GM and lung CFU using the late intervention protocol were similar to those obtained using the early intervention protocol (shown above). Grocott staining preparations showed an obvious focus of fungal infection (Figure 4B), and early and late intervention of posaconazole at 0.07 mg/mouse (Figure 4D and F, respectively) clearly reduced fungal infection.

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![FIGURE 1](image-url) **Kaplan-Meier analysis of survival proportions in Aspergillus-infected immunocompromised mice.** Survival curves of *A. fumigatus* (ATCC13073) (A) and (NCPF7100) (B) -infected immunocompromised mice treated with posaconazole intranasally (0.0028-0.35 mg/mouse, n = 8) once daily for 7 days. Body weights were measured daily and if body weight fell > 20% compared to that on day 1, the mice were regarded as dropout.
**FIGURE 2** Kinetics of biomarkers in *Aspergillus*-infected immunocompromised mice. Mice were dosed with hydrocortisone and cyclophosphamide to induce immunosuppression and neutropenia, and then infected intranasally with the suspension of *A. fumigatus* (ATCC13073). Mice were sacrificed on days 0, 1, 2, 3, 4, 6 and 8, and then bronchoalveolar lavage fluid (BALF), serum and lungs were collected for biomarker and CFU analysis. Each value is presented as mean±SEM (n = 3-6). CFU (A), Galactomannan (GM) in BALF (B), GM in serum (C), *Aspergillus* DNA in lung (D), Macrophage in BALF (E), neutrophils in BALF (F), malondialdehyde (MDA) in BALF (G), IFNγ in BALF (H) and IL-17 in BALF (I). Significant difference from day 0 (postinfection) value at *P* < .05 or **P** < .01 or ***P** < .001.

**FIGURE 3** Effects of intranasal posaconazole on fungal load in *Aspergillus*-infected immunocompromised mice. Posaconazole treated intranasally in early intervention (days 0-3; 0.014 and 0.07 mg/mouse) or late therapeutic intervention on (days 1-3; 0.014-0.35 mg/mouse), and mice were sacrificed 6 hours after the last dose of drug, and then bronchoalveolar lavage fluid (BALF), serum and lungs were collected for Galactomannan (GM) and CFU analysis. Each value is presented as mean±SEM (n = 6). Significant difference from vehicle control value at *P* < .05 or **P** < .01 or ***P** < .001.
As well as intranasal treatment, early intervention with orally treated posaconazole reduced GM in BALF and serum, and CFU in a dose-dependent manner with ID_{50} values of 0.051, 0.044 and 0.062 mg/kg, po, respectively (Figure S2). Late intervention with oral posaconazole also significantly reduced GM in BALF and serum and CFU with ID_{50} values of 0.094, 0.073 and 0.135 mg/kg, po, respectively (Figure S2), which were only slightly higher than those obtained by the early intervention.

3.5 Effects of intranasal posaconazole on infection-associated biomarkers in Aspergillus fumigatus (Af)-infected mice

Accumulation of macrophage and neutrophils and the levels of other markers such as MDA, IFN-γ and IL-17 in BALF were significantly reduced by early intervention of intranasal posaconazole and the effects were more potent than the late intervention (Figure S3A and B, Table 2).

| Dose (mg/mouse) | Early intervention | Late intervention |
|-----------------|--------------------|-------------------|
|                 | Vehicle           | 0.014             | 0.07             | 0.014           | 0.07            | 0.35             |
| IL-17 (pg/mL)   | 22.59 ± 3.18      | 15.61 ± 2.8       | 5.69 ± 0.23      | 19.14 ± 2.35    | 14.45 ± 1.14    | 8.59 ± 1.21      |
| [Inhibition %]^a| [31%]             | [75%]             | [15%]            | [36%]           | [62%]           |                  |
| MDA (μM)        | 1.14 ± 0.15       | 0.91 ± 0.09       | 0.47 ± 0.05      | 1.1 ± 0.09      | 0.82 ± 0.08     | 0.64 ± 0.09      |
| [Inhibition %]^a| [20%]             | [59%]             | [4%]             | [28%]           | [44%]           |                  |
| IFN-γ (pg/mL)   | 9.94 ± 1.48       | 3.87 ± 0.58       | 1.99 ± 0.13      | 8.05 ± 0.78     | 4.77 ± 0.39     | 3.21 ± 0.41      |
| [Inhibition %]^a| [61%]             | [80%]             | [19%]            | [52%]           | [68%]           |                  |

^aThe value is compared with vehicle control.
This study demonstrated for the first time that intranasal posaconazole treatment improved survival rates in itraconazole-susceptible (Af) and -resistant (AfR) A. fumigatus-infected immunocompromised mice in a similar fashion to oral treatment. Furthermore, we analysed A. fumigatus infection-associated biomarkers more quantitatively, and the data demonstrated potent inhibition of fungal load by intranasally treated posaconazole.

For the Af-infected mice once daily, intranasal treatment with posaconazole demonstrated dose-dependent improvement of survival rates with an ED$_{50}$ value of 0.755 mg/kg, which was similar potency to that obtained using oral treatment (ED$_{50}$: 0.775 mg/kg). In a strain AfR, oral treatment did not affect survival rate up to 5 mg/kg, po (33% survival rate). In contrast, intranasal posaconazole achieved 75% survival rate at 14 mg/kg, which is equivalent to that observed at 2.8 mg/kg in Af strain (88% survival). Thus, intranasal posaconazole was effective and only fivefold weaker in AfR in vivo. In the in vitro microdilution test, posaconazole was 17-fold weaker in AfR strain than Af strain, but posaconazole indeed showed MIC (complete inhibition) at higher doses. This suggests that if the local concentration of a triazole is kept in the therapeutic range, it is possible to inhibit the fungus growth of a resistant strain. Thus, the potential to maintain high local concentrations is an advantage of topical treatment, and so provide a new opportunity to treat triazole-resistant A. fumigatus infection$^{27,28}$ in the clinic.

In this study, we investigated not only survival rates but also several biological markers such as CFU fungal load, the levels of GM and other infection-associated biomarkers including cytokines. Intranasal posaconazole inhibited GM and CFU in a dose-dependent manner and also induced almost complete inhibition at higher doses, although oral treatment did not achieve complete inhibition (Figure S2A and C). In addition, we observed the marked drop in fungal burden and galactomannan in serum and BALF, which was associated with intranasal posaconazole treatment. As shown in kinetics of neutrophils in Figure 2F, mice showed strong neutropenic condition on Day 1 postinfection (=3 days after cyclophosphamide injection), but the neutrophil content in BALF was gradually increased with Aspergillus infection. Probably, posaconazole reduced fungus to certain level directly (even not completely) first 2 days, and recovered neutrophils easily eliminated rest of fungal body. Thus, the threshold effects might be a reflection of neutrophil recovery in animal. However, as we did not observe posaconazole dose-dependent induction or quick recovery of neutrophils in posaconazole-treated mice, at least we do not think posaconazole eliminated fungus by increased number of neutrophils.

The complete inhibition by intranasal posaconazole was also confirmed by histology where fungus was visualised by silver staining (Figure 4). Thus, greater maximum inhibition was obtained by intranasal than oral treatment over the doses ranges studied here, but assessed by calculated ID$_{50}$ values, intranasal treatment was less potent vs those fungal biomarkers than oral treatment.

In our temporarily neutropenic model, marked production of IL-17 and IFN-γ was observed in BALF after A. fumigatus infection (Table 2 and S1). Mirkov et al.$^{29,30}$ also demonstrated changes in pro-inflammatory cytokines such as IFN-γ and IL-17 in A. fumigatus-infected mice and rats. Both cytokines, which are components of host anti-fungal defence to A. fumigatus infection, were increased over time up to day 8 postinfection. Although intranasal posaconazole showed inhibitory activity of these cytokines in A. fumigatus-infected mice (Table 2), this does not mean treatment inhibited host innate immunity but is probably a secondary consequence of inhibition of fungus growth by posaconazole.

Aspergillus fumigatus infection was reported to increase MDA levels caused by the oxidant-antioxidant imbalance in neutrophic mice lung homogenates.$^{31}$ We also found that MDA was increased during A. fumigatus infection. MDA was highly induced just after infection probably as a part of host defence, and then gradually increased overtime, peaking between days 4 and 6, when fungal biomarkers also peaked (Figure 2G). As MDA kinetics resembled fungal biomarkers, the change in oxidative stress was apparently associated with A. fumigatus growth, suggesting that MDA will be an interesting marker of A. fumigatus infection. The elevated MDA levels also responded well to intervention with intranasal posaconazole.

The lack of pharmacokinetic measurement of posaconazole concentrations in plasma and ELF is a limitation of this study. It is therefore difficult to interpret the different effects of two administration routes, although we found that both oral and intranasal treatment improved survival proportions similarly. However, we carefully optimised the intranasal volume (35 μL), and as previously reported,$^{19}$ 35 μL of intranasal volume is expected for 60% of inoculum to deposit in lung. This means 40% of inoculum or treated posaconazole might be swallowed or stayed in nasal cavity, and therefore it cannot be ruled out that intranasally treated posaconazole was exposed systemically via gastrointestinal route or nasal route. Seyedmousavi et al.$^{20}$ who evaluated the pharmacokinetic parameters of posaconazole in immunosuppressed mice, suggested that high plasma protein binding may explain the reason why effective local concentrations can be achieved, even at the low dose. In addition, it has been suggested that the more hydrophobic posaconazole allows an even higher intracellular uptake than voriconazole.$^{32}$ This would suggest that posaconazole would be the better candidate for topical treatment than voriconazole. However, we report here that oral posaconazole was more potent than intranasal posaconazole on biomarkers based on the ID$_{50}$ values, and so are unlikely to be ideal for topical treatment.

We demonstrated that intranasally dosed posaconazole produced potent inhibition of A. fumigatus-induced inflammation, fungal load and improvement of survival rate. These findings suggest that topical administration of posaconazole is a potential option for the treatment of A. fumigatus infection in humans with higher efficacy and less toxicities.

**CONFLICT OF INTEREST**

KI, PS, GR are employees and (co) founders of Pulmocide Ltd. YK received funding from Pulmocide Ltd. Other authors have nothing else to declare.
AUTHOR CONTRIBUTIONS
This study was designed by KI, PS, GR and YK, and GK, TN, YN, YS and YK conducted in vivo and in vitro studies. The manuscript was drafted by KI, PS, GK, TN and YK, and was reviewed by all authors.

ETHICS APPROVAL
All animal studies were approved by the Ethics Review Committee for animal Experimentation of Nihon University. All Aspergillus fumigatus studies were approved by the Microbial safety Management Committee of Nihon University School of Pharmacy (E-H25-001).

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the supporting information tab for this article.

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