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Murine airway histology and intracellular uptake of inhaled amorphous itraconazole

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Abstract

Aerosolization of amorphous itraconazole may be a safe and effective method of pulmonary delivery. Our objective was to evaluate the histologic effects, immunogenic potential, and cellular uptake of aerosolized amorphous itraconazole. Mice received amorphous itraconazole (30 mg/kg), excipient placebo, or saline control by nebulization every 12 h for up to 12 days. Broncho-alveolar lavage (BAL) and formalin fixation of both lungs were conducted. BAL supernatant was assayed for IL-12 by ELISA, and cellular components were analyzed by high performance liquid chromatography–mass spectroscopy. Coronal sections of the entire lung were stained, viewed by light microscopy, and the Cimolai histopathologic inflammatory score was obtained for each lobe. No evidence of bronchiolar, peribronchiolar or perivascular inflammation was found in any treatment group, nor were epithelial ulceration or repair observed. The Cimolai histopathologic scores for amorphous itraconazole, excipient, and saline control on days 3 and 8 did not differ between groups. ELISA analysis showed no cytokine induction of IL-12. Itraconazole was detected within cells collected from BAL fluid on days 1, 3, 8 and 12. Aerosolized administration of amorphous itraconazole or excipients does not cause inflammation or changes in pulmonary histology and are not associated with pro-inflammatory cytokine production.

Keywords: Itraconazole; Aerosolized delivery; Pulmonary histology

1. Introduction

Invasive aspergillosis primarily occurs in immunocompromised patients via inhalation of conidia into the lungs. Over the last two decades the incidence of infections due to this opportunistic pathogen has increased 3–4-fold with an attributable mortality rate ranging between 60 and 90% in patients with hematologic malignancies (Baddley et al., 2001; Kontoyiannis et al., 2003; Marr et al., 2002; Wald et al., 1997). Unfortunately, current treatment strategies for invasive aspergillosis are suboptimal and are limited by adverse effects and drug interactions due to collateral toxicities with mammalian cells.

Because the primary route of entry of *Aspergillus* is through the lungs, attention has recently been focused on the pulmonary delivery of antifungal agents (Perfect et al., 2004). Aerosolized administration of amphotericin B deoxycholate as well as lipid

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amphotericin B formulations has been used for prophylaxis and treatment of invasive aspergillosis (Boots et al., 1999; Drew et al., 2004; Koizumi et al., 1998; Lambros et al., 1997; Monforte et al., 2003). However, these formulations are not designed for aerosolized administration, and toxicity has been reported with the deoxycholate and lipid formulations of amphotericin, including concerns of exogenous lipoid pneumonia and fibrosis of the lung tissue (Spickard and Hirschmann, 1994). Furthermore, despite efficacy in lung transplant recipients (Drew et al., 2004; Palmer et al., 2001), inhaled amphotericin B was shown to be ineffective as prophylaxis in patients with prolonged neutropenia following chemotherapy or autologous bone marrow transplantation (Schwartz et al., 2000).

The triazole itraconazole (ITZ) has a broad spectrum of activity including *Aspergillus* species (Chiller and Stevens, 2000). However, poor oral bioavailability, variable absorption and gastrointestinal toxicity due to the hydroxypropyl-β-cyclodextrin component of the oral solution limit itraconazole to a second or third line treatment option for invasive fungal infections (Allendoerfer et al., 1995; Kapsi and Ayres, 2001). Aerosolized administration of ITZ has been proposed as a means to improve localized delivery of this agent to the primary site of infection for the treatment and prophylaxis against invasive aspergillosis.

Spray freezing into liquid is an engineering process producing nanostructured particles (<1 μm in diameter) that capable of drug deposition in the alveolar space (Rogers et al., 2002). Recent studies in our laboratories have demonstrated high lung concentrations and a survival benefit of aerosolized nanostructured ITZ formulations as prophylaxis compared to commercially available ITZ oral solution (Hoeben et al., 2006; McConville et al., 2006). However, histologic data and intracellular drug disposition following inhalation of ITZ have not been evaluated. The objective of this study was to evaluate the safety of an amorphous nanostructured ITZ formulation delivery by aerosolization to the lungs through assessment of changes in pulmonary histology. Measurement of a soluble inflammatory mediator (IL-12) and phagocytic uptake of ITZ by cells collected following bronchoalveolar lavage, including pulmonary macrophages, were also evaluated.

2. Methods

2.1. Production of amorphous nanostructured ITZ

Amorphous nanostructured ITZ was produced using ITZ powder (Hawkins Chemicals, Minneapolis, MN) and the spray freezing into liquid (SFL) particle engineering process (Rogers et al., 2002). A solution of ITZ, polysorbate 80 and poloxamer 407, in a 1:0.75:0.75 ratio in acetonitrile with 4% (w/w) dichloromethane as co-solvent, was atomized through a 63 μm poly-ether-ketone nozzle (Upchurch Scientific, Oak Harbor, WA) via an HPLC pump at 20 mL/min below the surface of liquid nitrogen. The frozen microparticles were separated from the liquid nitrogen, the solvent was removed by lyophilization, and the dried powders were stored under vacuum. The physical characteristics of this formulation have previously been reported (McConville et al., 2006).

2.2. Aerosolization of ITZ, excipient placebo and saline control

Female ICR mice (Harlan Sprague–Dawley, Indianapolis, IN) weighing 20–25 g received either aerosolized amorphous ITZ, excipient placebo, or saline control for up to 12 days using a previously described dosing chamber (McConville et al., 2005). The saline control consisted of a 0.85% NaCl solution used for dispersion of the excipient and drug formulations. The excipient placebo consisted of 15 mg/mL polysorbate 80 and 15 mg/mL poloxamer 407 dissolved in 0.85% NaCl. A 20 mg/mL dispersion of amorphous ITZ was formed in 4 mL of 0.85% NaCl by first wetting the solution followed by sonication for 1 min prior to dosing. Amorphous ITZ ($N = 22$), excipient placebo ($N = 10$), or saline control ($N = 10$) were administered via a 20-min aerosolization every 12 h with an Aeroneb Pro micro pump nebulizer (Aerogen, Inc., Mountain View, CA) situated at the inlet of the dosing chamber. This protocol was approved by the Institutional Animal Care and Use Committees at the University of Texas Health Science Center at San Antonio.

2.3. Histology

Histologic changes in lung tissue of mice following 3 and 8 days of aerosolized administration of amorphous ITZ were compared to those receiving excipient placebo or saline control. Five animals were included in each group at each time point. Mice were euthanized and 10% (v/v) formaldehyde was instilled into the lungs through a canula placed inside a small incision in the trachea. Lungs were then harvested and placed into 10% (v/v) formaldehyde followed by processing and embedding into paraffin wax. Coronal sections of the entire lung were stained and viewed by light microscopy. The Cimolai histopathologic inflammatory score (0–26, least to most severe histologic response based on degree and location of inflammation) was obtained for each lobe (Cimolai et al., 1992). Evidence of bronchiolar ulceration and tissue repair were also assessed. Scores were compared by analysis of variance.

2.4. Bronchoalveolar lavage (BAL) fluid collection, intracellular ITZ, and cytokine assay

BAL fluid was collected following euthanization from 24 mice (12 aerosolized ITZ, 6 excipient control, and 6 saline control; 3 animals per group per time point). Lungs were lavaged by cannulization of the trachea and instillation and removal of 0.75 mL of phosphate buffered saline (PBS) for a total of three washes. Cells were collected from the BAL fluid by centrifugation and washed three times in PBS. Aliquots of the cells were viewed by light microscopy (Axioskop 2 plus, Carl Zeiss International, Thornwood, NY) to confirm the presence of pulmonary macrophages. ITZ was extracted and concentrations measured by high performance liquid chromatography–mass spectrometry (HPLC-MS) (Gubbins et al., 1998). Briefly, the pellets containing the cells were redispersed in 0.25 mL phosphate buffered saline (pH 7.6, 25 °C) and vortexed for 30 s. Zinc sulfate and barium hydroxide were added to each sam-

ple in 50 μL aliquots and vortexed for 30 s. One milliliter of acetonitrile was added and vortexed for 1.5 min, centrifuged at 3000 rpm for 15 min, after which, the supernatant was transferred to a separate vial and dried at 60 °C under a stream of dry nitrogen gas. After drying to evaporation, the extracted drug was redispersed in 0.25 mL of mobile which consisted of 62% acetonitrile and 38% water. The extracted sample was then manually fractionated using HPLC on a Shimadzu LC-10vp (Shimadzu Scientific Instruments, Columbia, MD) with an Alltima 5 μ 250 mm × 4.6 mm (Alltech Associates, Deerfield, IL) column held constant at 37 °C. The injection volume was 100 μL and a flow rate of 1 mL/min with an ITZ retention time of 17.7 min and a peak width of 0.75 min based on standard solutions. The standard solutions consisted of ITZ concentrations of 1.2 μg/mL and 12 ng/mL which were subjected to fractionation and MS analysis. Drug fractions were collected between 17 and 18.5 min and then run on MS for qualitative confirmation of ITZ presence. The samples were analyzed by mass spectroscopy at the Center for Research and Environmental Disease at the University of Texas at Austin. For cytokine analysis IL-12p70 concentrations were measured in the BAL supernatant in duplicate by a murine specific enzyme-linked immunosorbent assay (ELISA) (Quantikine-M, R&D Systems, Minneapolis, MN) according to the manufacturers instructions. Differences in IL-12p70 concentrations were assessed by analysis of variance with Tukey's post-test for multiple comparisons.

3. Results

3.1. Histology

Fixed lungs were sectioned and viewed for pulmonary damage and/or inflammation secondary to amorphous ITZ or excipient inhalation. Representative sections are shown in Fig. 1 from the mice administered saline control (A and B), excipient placebo (C and D), or amorphous ITZ (E and F). In each group, alveoli remained intact with no evidence of damage, inflammation or cell migration. Airways remained clear and displayed a single cell layer, indicative of non-inflamed airways. Furthermore, no bronchiolar, peribronchiolar or perivascular inflammation, or any evidence of epithelial ulceration or repair was observed in any group. The Cimolai histopathologic inflammatory scores (mean ± S.D.) for amorphous ITZ, excipient placebo, and saline control following 3 days of administration were 2.4 ± 1.1, 2.5 ± 1.3, and 3.0 ± 1.2 ($p > 0.05$), respectively, indicating minimal inflammation. Similarly, at day 8 Cimolai scores were 3.3 ± 0.8, 2.7 ± 0.8, and 3.2 ± 0.8 ($p > 0.05$), respectively, demonstrating no increase in inflammatory changes over time with repeated dosing of amorphous ITZ or the excipient placebo consisting of polysorbate 80 and poloxamer 407. Interestingly, vascular congestion and edema was equal in all groups and likely secondary to termination by CO_2 narcosis. To demonstrate that the edema was secondary to CO_2 narcosis, a second experiment was conducted exposing mice ($n = 3$ per group) to 8 days of aerosolized saline control or amorphous ITZ. Animals were sacrificed using isoflurane and the entire lung processed for pathologic review. In both the control and amorphous ITZ groups

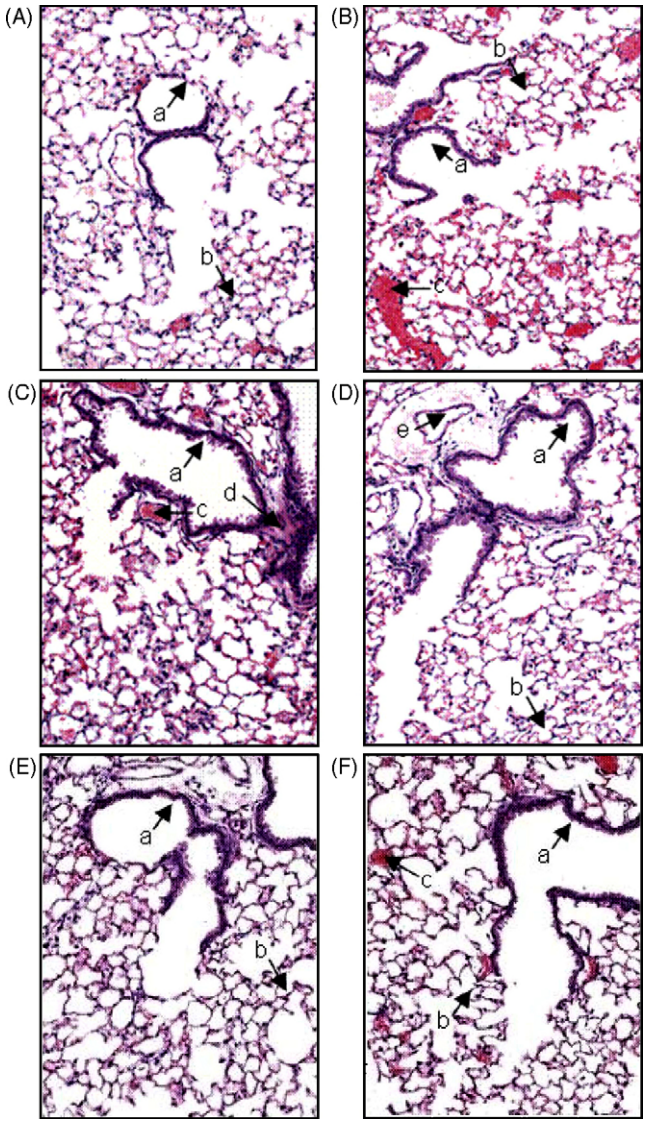

Fig. 1. Representative histology sections from the saline control ((A) day 3; (B) day 8), excipient placebo ((C) day 3; (D) day 8) and amorphous ITZ composition groups ((E) day 3; (F) day 8). Structures noted in the histology samples are labeled: airways (a), alveolar spaces (b), capillaries (c), lymph tissue (d) and arteriols (e). Magnification 20×.

the lungs had normal alveolar and interstitial histology without evidence of inflammation or mucosal ulceration within the airways (Cimolai score 1.5 and 1.3, respectively). Only minimal focal vascular congestion was noted equally in both groups.

3.2. Interleukin-12 cytokine analysis

Supernatant from the BAL samples was analyzed for the presence and elevation of IL-12 to determine if inflammation was elicited from pulmonary dosing of the amorphous ITZ composition or the excipient placebo. As shown in Fig. 2, IL-12 concentrations within BAL supernatant from mice that received aerosolized administration of amorphous ITZ, excipient placebo, or saline for 1, 3, or 8 days were negligible and similar to the negative control ($p > 0.05$). Similarly, no IL-12 was detected after 12 days of aerosolized amorphous ITZ.

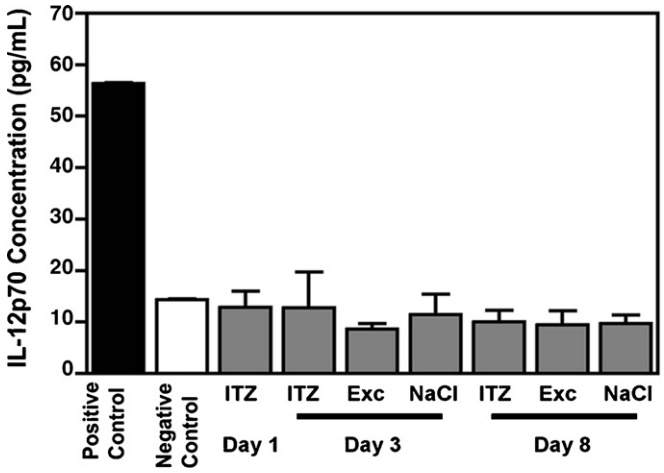

Fig. 2. Mean IL-12p70 concentrations measured via ELISA assay of the positive control, negative control and BAL supernatants of sacrificed mice. ITZ, itraconazole; Exc, excipient control; NaCl, saline control.

An increase in the IL-12 would have suggested an underlying inflammation not observed during histologic analysis (Ichinose et al., 2005). However, the ELISA results suggest that there was no induction of a localized inflammatory response within the lungs due to the inhalation of amorphous ITZ or the excipients polysorbate 80 and poloxamer 407.

3.3. Cellular uptake of amorphous ITZ

Microscopic analysis of cells collected from BAL fluid was conducted to ensure the presence of pulmonary macrophages. Macrophages were readily distinguishable from other cells through visualization of the phagosomes (Fig. 3). The remaining cells underwent drug extraction, chromatographic separation and mass spectroscopy for qualitative assessment of intracellular ITZ concentrations. ITZ was present within cells collected from the BAL fluid from mice administered amorphous ITZ (days 1, 3, 8 and 12). Recognition of foreign particles by pulmonary macrophages is dependent on hydrophilicity, size and surface charge, with hydrophobic and/or negatively charges particles in the size range of 0.2–10 μm preferentially phagocytized (Makino et al., 2003). Thus, amorphous ITZ particles, which are hydrophobic and within this range in diameter (McConville et al., 2006), are ideal for phagocytosis by pulmonary macrophages.

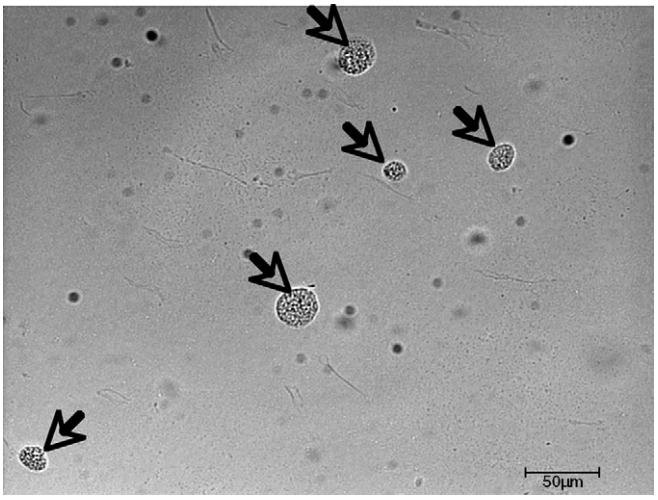

Fig. 3. Representative micrograph showing the presence of macrophages within the BAL pellet following centrifugation of BAL fluid and washing with phosphate buffered saline.

4. Discussion

Previous work from our group has demonstrated high concentrations of ITZ within the lung following aerosolized administration of amorphous ITZ and that this strategy is effective as prophylaxis against invasive pulmonary aspergillosis (Hoeben et al., 2006; Vaughn et al., 2006). However, the effects of this strategy on the lungs were unknown. The primary objective of this study was to evaluate the immunogenic and histologic effects of aerosolized amorphous ITZ. To this end, the Cimolai histopathologic inflammatory score was utilized to quantify the histologic changes (Cimolai et al., 1992). This scoring index, ranging between 0 and 26 from least to severe inflammation, is based on histologic findings including the quantity and quality of peribronchiolar and peribronchial infiltrates, luminal exudates, perivascular infiltrates, and parenchymal pneumonia. Both aerosolized amorphous ITZ and excipient placebo did not result in increased inflammation, inflammatory cell migration, or tissue damage compared to controls. Histologic sections demonstrated clear and undamaged bronchial/bronchiolar regions and alveolar spaces without evidence of immune cell infiltration. While perivascular edema was noted in all samples from animals sacrificed by CO_2 narcosis, normal alveolar and interstitial histology was observed in repeat studies using isoflurance for euthanization.

To our knowledge, no previous study has evaluated the safety of aerosolized ITZ. However, others have assessed the effectiveness and safety of aerosolized amphotericin B. Adverse effects with this strategy have included cough, dyspnea, bronchospasm, nausea, and vomiting with aerosolized administration of amphotericin B formulations (Drew et al., 2004; Dubois et al., 1995; Palmer et al., 2001). These effects may primarily be due to the deoxycholate constituent used to form the colloidal suspension. In vitro studies have shown a dose-dependent inhibition of surfactant function that was not observed with either amphotericin B alone or a lipid formulation (Griese et al., 1998; Ruijgrok et al., 2001). Furthermore, aerosolized amphotericin B lipid complex was demonstrated to be better tolerated than amphotericin B deoxycholate in lung transplant recipients with no evidence of histopathologic abnormalities or lipoid pneumonia (Drew et al., 2004; Palmer et al., 2001). These observations highlight the importance of the excipients used in formulations for aerosolized delivery. The excipients in the amorphous ITZ formulation used in this study, polysorbate 80 and poloxamer 407, did not elicit an immune response or cause changes in pulmonary histology. Polysorbate 80 is contained within several commercial and experimental formulations and is well established as a safe surfactant for pulmonary administration (Williams and Liu, 1999). However, no commercial product containing poloxamer 407 has

been approved by the FDA, nor has this excipient been studied for pulmonary delivery. While the effects of these agents on surfactant were not evaluated in this study, no adverse effects on surfactant function are expected since each is a non-ionic surfactant.

In addition to the histology results, no elevations in IL-12 were observed in BAL fluid from mice administered amorphous ITZ, excipient placebo, or control, suggesting a lack of local immunogenic potential with aerosolized delivery of either the active drug or the excipients. IL-12 is released by antigen-presenting cells of the innate immune system and elicits a pro-inflammatory Th1 lymphocyte response. While pro-inflammatory cytokines are typically measured in the serum, local sensitization and a strong airway inflammatory response may not always elicit systemic inflammation. As demonstrated by Repa et al. (2004), aerosolization of a known allergen to mice (birch pollen extract) resulted in low systemic inflammatory response despite strong airway inflammation as measured by elevated cytokine concentrations within BAL fluid. Furthermore, the utility of measuring IL-12 in BAL fluid as a marker of inflammation has been previously demonstrated. Ichinose et al reported significant increases in IL-12 concentrations (range 200–900 pg/mL) measured in BAL supernatants along with elevations in other pro-inflammatory markers taken from mice following intratracheal instillation of Asian yellow dust, an agent known to cause adverse respiratory effects in humans (Ichinose et al., 2005). The absence of an increase in IL-12 following aerosolization of excipients in our study was not unexpected. A prior study demonstrated no increases in IL-12 or other cytokines following incubation of macrophages with solid lipid nanoparticle formulations containing both polysorbate 80 and poloxamer 407 (Scholer et al., 2001). These results demonstrating no increases in the pro-inflammatory cytokine IL-12 within BAL fluid following aerosolized amorphous ITZ or excipient placebo are in agreement with the data from histology. Measurement of other pro- and anti-inflammatory cytokines was not performed but may be warranted in future studies.

In the current study, ITZ was detected within cells recovered from BAL fluid, including pulmonary macrophages, thus demonstrating intracellular uptake following aerosolized delivery. A primary host defense against invasive pulmonary fungal infections (e.g., invasive aspergillosis) is uptake of conidia by alveolar macrophages. However, phagocytosis by alveolar macrophages does not always result in effective killing of fungal spores or prevent germination into invasive hyphae. Corticosteroids have been shown to impair the production of reactive oxidant intermediates and the killing of *A. fumigatus* conidia by alveolar macrophages without altering phagocytosis (Philippe et al., 2003). The ability of ITZ to be taken up by alveolar macrophages presents a unique opportunity for augmentation of host defense with drug treatment through the presence of antifungal activity within the cells that internalize fungal spores. Delivery of ITZ by aerosolization to a primary site of infection and intracellular uptake by pulmonary macrophages may aid in preventing invasive pulmonary fungal infections even in the presence of agents known to inhibit intracellular killing of fungal spores. Indeed, a previous study has demonstrated improved survival with aerosolized amorphous ITZ in mice immunosuppressed with cortisone acetate following pulmonary challenge with *Aspergillus* (Hoeben et al., 2006).

In conclusion, the results of this study demonstrate that aerosolized administration of amorphous ITZ or its excipients for up to 12 days do not appear to be immunogenic and do not cause changes in pulmonary histology. Furthermore, the ability to detect ITZ within cells collected from BAL fluid, including pulmonary macrophages, raises the possibility that this strategy may augment host defenses and help prevent invasive fungal infections in the presence corticosteroid induced immune suppression.

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