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Dose tolerability of chronically inhaled voriconazole solution in rodents

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ABSTRACT

Invasive pulmonary aspergillosis (IPA) is a fungal disease of the lung associated with high mortality rates in immunosuppressed patients despite treatment. Targeted drug delivery of aqueous voriconazole solutions has been shown in previous studies to produce high tissue and plasma drug concentrations as well as improved survival in a murine model of IPA. In the present study, rats were exposed to 20 min nebulizations of normal saline (control group) or aerosolized aqueous solutions of voriconazole at 15.625 mg (low dose group) or 31.25 mg (high dose group). Peak voriconazole concentrations in rat lung tissue and plasma after 3 days of twice daily dosing in the high dose group were 0.85 ± 0.63 μg/g wet lung weight and 0.58 ± 0.30 μg/mL, with low dose group lung and plasma concentrations of 0.38 ± 0.01 μg/g wet lung weight and 0.09 ± 0.06 μg/mL, respectively. Trough plasma concentrations were low but demonstrated some drug accumulation over 21 days of inhaled voriconazole administered twice daily. Following multiple inhaled doses, statistically significant but clinically irrelevant abnormalities in laboratory values were observed. Histopathology also revealed an increase in the number of alveolar macrophages but without inflammation or ulceration of the airway, interstitial changes, or edema. Inhaled voriconazole was well tolerated in a rat model of drug inhalation.

1. Background

Invasive pulmonary aspergillosis (IPA) is an opportunistic infection that primarily affects immunocompromised individuals with the highest infection rates in patient populations with hematologic malignancies, hematopoietic stem cell transplant recipients, and those undergoing solid organ transplantation (Lin et al., 2001; Denning, 1998). The causative epidemiology of IPA is also changing to more serious *Aspergillus* spp. with mortality rates up to 75% in some cases and substantial healthcare costs per case (Maschmeyer, 2006; Cornet et al., 2002; Dixon et al., 2004; Maschmeyer et al., 2007). The serious effects of IPA have prompted investigations to improve therapeutic options for this disease (Patterson, 2005; Yang et al., 2008c).

In 2002, Herbrecht and colleagues published the results from a multi-center randomized controlled clinical trial that compared the then standard of care, intravenous (IV) amphotericin B deoxycholate, and IV voriconazole with the option to switch patients to oral (PO) voriconazole. This study established voriconazole as the first-line therapeutic option in the treatment of IPA (Herbrecht et al., 2002; Walsh et al., 2008). Although this study changed the treatment paradigm for IPA, the mortality rates over 12 weeks remained high (29.2% and 40.3% for voriconazole and amphotericin B, respectively), prompting researchers to investigate different treatment modalities and approaches to the therapeutic management of IPA (Cornet et al., 2002; Maschmeyer et al., 2007). Targeted antifungal delivery to the lung was proposed to cause high drug concentrations at the primary site of infection leading to increased efficacy and better patient outcomes but with lower systemic exposure and subsequent

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decreased rates of adverse events associated with the antifungal agent.

One of the promising therapeutic approaches included the prophylactic inhalation of antifungals, including aerosolized amphotericin B formulations, in patients at high risk for IPA (Schwartz et al., 1999; Mohammad and Klein, 2006; Playford et al., 2006a; Playford et al., 2006b). The various formulations of amphotericin B have been investigated by nebulization, including the deoxycholate (Fungizone®), lipid complex (Abelcet®), or liposomal (Ambisome®) forms and have led to inconsistent reports of prophylactic efficacy and patient tolerability leading to no unified standard for aerosolized amphotericin B prophylaxis (Dummer et al., 2004; Monforte et al., 2003; Cohen, 1998). Pre-clinical testing of aerosolized itraconazole in animal models have also been investigated and reviewed in efforts to reduce complications and inconsistencies associated with inhaled amphotericin B (Purvis et al., 2006; Yang et al., 2008a,b,c). These researchers administered nano-suspensions of engineered crystalline and amorphous itraconazole particles to mice to establish pharmacokinetic and efficacy profiles for inhaled nanoparticulate itraconazole. Itraconazole was retained in lung tissue for several hours with very little drug distributing to the blood after administration with favorable pharmacokinetic parameters (Mcconville et al., 2006; Vaughn et al., 2006; Yang et al., 2008b). In addition, 12-day survival rates in mice infected with *A. flavus* and *A. fumigatus* treated daily with inhalations of itraconazole were significantly better than a positive control and were 50–80% and 35%, respectively (Alvarez et al., 2007; Hoeben et al., 2006). Inhaled nanoparticulate itraconazole was also well tolerated by evaluation of histopathological lung tissue sections (Vaughn et al., 2007).

Although nanoparticulate itraconazole suspensions had encouraging results, voriconazole is the first-line therapeutic agent for the treatment of IPA due to improved survival benefits and with an expanded antifungal activity compared to itraconazole (Gabardi et al., 2007). Voriconazole is generally well tolerated but with therapy-limiting hepatotoxicity as measured by elevations in liver function tests, having been correlated with peak plasma concentrations (Husain et al., 2007; Scott et al., 2007). Systemic administration of voriconazole has led to variable pharmacokinetic properties and poor lung tissue distribution resulting in-part from non-linear pharmacokinetics in adults resultant from saturable metabolism (Scott et al., 2007; Walsh et al., 2004; Theuretzbacher et al., 2006). Through targeted drug delivery to the lungs, inhaled voriconazole could offer higher tissue concentrations than possible following systemic drug delivery and lead to improvements over both inhaled amphotericin B and itraconazole as prophylaxis against IPA.

The IV form of voriconazole, a powder for injection (Vfend® IV), contains voriconazole with sodium sulfobutyl ether-β-cyclodextrin in an inclusion complex to improve the aqueous solubility of the poorly water soluble active agent. High lung tissue and plasma concentrations with a lung tissue to plasma concentration ratio of 1.4 to 1 were observed in a single and multiple dose pharmacokinetic study of inhaled voriconazole in mice (Tolman et al., 2009a). In addition, this inhaled formulation of voriconazole as prophylaxis resulted in improved survival in a murine model of IPA (Tolman et al., 2009b). In these studies, phenotypic evaluation of murine behavior and outward appearance suggested inhaled voriconazole was well tolerated. The purpose of this study was to conduct a more thorough analysis which was performed with inhaled voriconazole administered twice daily (BID) to assess for hepatic, electrolyte, renal, or erythrocyte or histiocyte abnormalities as suggested in the prescribing information for Vfend® IV (Roerig Division of Pfizer Inc., 2006). Organs were also evaluated for histopathological changes at the site of administration and absorption, metabolism and elimination. It is postulated that inhaled voriconazole is well tolerated and comparable to a negative inhaled control group.

2. Materials and methods

2.1. Materials

Vfend® IV (Pfizer Inc., New York, NY, USA), voriconazole, and sulfobutyl ether-β-cyclodextrin, Captisol® were generously supplied by CyDex Pharmaceuticals, Inc. (Lenexa, KS). The following items were purchased from the respective suppliers: sterile water for injection (SWFI) and normal saline from Cardinal Health (Dublin, OH); sodium tetraborate decahydrate, boric acid, sodium acetate trihydrate, and neutral buffered formalin 10% solution from Sigma–Aldrich, Inc. (St. Louis, MO); acetic acid from Sigma–Aldrich Laborchemikalien GmbH (Seelze, Germany); heparin sodium injection, USP from Baxter Healthcare Corporation (Deerfield, IL); Lavender-topped (LT) BD Microtainer® Tubes with K2E (K2EDTA) and Red-topped (RT) BD Microtainer® No additive Tubes from Becton, Dickinson and Company (Franklin Lakes, NJ); and HPLC grade solvents including ethyl acetate from Spectrum Chemical Manuf. Corp. (Gardena, CA); acetonitrile from Fisher Scientific (Fair Lawn, NJ); and methanol from EMD Chemicals Inc. (Gibbstown, NJ).

2.2. Study design

Male and female Sprague–Dawley rats, Harlan Sprague Dawley, Inc. (Indianapolis, IN), with an average mass of 250 g at the beginning of the study, were caged separately with free access to food and water. Prior to dosing, animals that would receive aerosolized treatments were acclimatized for up to 20 min twice daily over 3 days to rodent nose-only restraint systems, Battelle Toxicology Northwest (Richland, WA). All animals were handled and maintained in accordance with The University of Texas at Austin Institution Animal Care and Use Committee (IACUC) guidelines and in accordance with the American Association for Accreditation of Laboratory Animal Care guidelines.

An isotonic drug solution was prepared with Vfend® IV reconstituted and diluted with SWFI to a voriconazole concentration of 6.25 mg/mL as described previously (Tolman et al., 2009a). For dose tolerability analysis, 45 male and 45 female rats were randomly divided into three treatment groups: High-dose (HD) that received 31.25 mg of drug in solution nebulized over 20 min; Low-dose (LD) that received 15.625 mg of drug in solution nebulized over 20 min; and Control (C) that received 5 mL of normal saline over 20 min. Another group of 20 rats, designated No Treatment (NT), received no treatment but were housed with free access to food and water and used for establishment of normal laboratory value ranges for statistical comparison. A separate group of eight male rats per group were used for pharmacokinetic evaluation of peak and trough voriconazole concentrations in lung tissue and plasma on day 3. Treatments were administered using an in-line Aeroneb® Pro micro pump nebulizer from Aerogen, Inc. (Mountain View, CA) with a nose-only dosing chamber with an air-flow rate of 1 L/min through the chamber. Treatments lasted for up to 20 min and all solutions were nebulized to dryness. Treatments were administered BID beginning at 08:00 and 16:00 and continued for up to 21 days with no additional treatments through day 28. Animals were sacrificed on days 7, 14, 21, and 28 by isoflurane narcosis. NT animals were euthanized on day 28, after having received no treatments, by isoflurane narcosis. Animals designated for pharmacokinetic analysis on day 3 were euthanized by CO_2 narcosis. Euthanasia was ensured by exsanguination via cardiac puncture followed by thoracotomy.

2.3. Plasma and tissue extraction for pharmacokinetic analysis

On day 3, whole blood collected via cardiac puncture was placed into heparinized vials and centrifuged at 9000RPM

for 15 min using a Microfuge® 18 Microcentrifuge from Beckman Coulter (Fullerton, CA). Plasma was collected into clean vials and frozen at −20 °C until analysis. Lungs were excised and cleaned from external lymphatic, connective, and airway tissue, placed in a clean vial, and frozen at −20 °C until analysis.

Calibration standards, plasma, and homogenized lung samples were analyzed using similar methods to those previously published (Tolman et al., 2009a; Pascual et al., 2007; Lutsar et al., 2003). Briefly, voriconazole was extracted from plasma samples through a series of acetonitrile extractions with fluconazole as an internal standard followed by solvent evaporation and reconstitution with acetonitrile. For lung analysis, lung tissue was homogenized with 2 mL of normal saline per gram wet lung weight, 0.2 M borate buffer (pH 9.0) was added, followed by three ethyl acetate extractions, solvent evaporation, and reconstitution with acetonitrile. A 4000 Q TRAP mass spectrometer (Applied Biosystems, Foster City, CA) coupled with an online HPLC (Shimadzu, Columbia, MD) was used to analyze the samples. 10 μl samples were injected into Shimadzu Prominence UFLC system equipped with an Restek C18 (4.6 × 50 mm, 5 μm, 110 Å) column and eluted with mobile phase A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) by a gradient of 20%B for 0.5 min, then 20–50%B over 0.5 min, followed by 50%B for 3 min, at a flow rate of 1 ml/min. Sample was directly eluted from the column into the electrospray ion (ESI) source of 4000 QTRAP. The heated nebulizer of 4000 QTRAP was set at 700 °C, the declustering potential (DP) at 40. The Multiple Reaction Monitoring (MRM) scan experiment with unit resolution for Q1 and low resolution for Q3 was used to quantify voriconazole content. The MRM transitions were set as follow, *Q*1 = 350.2, *Q*3 = 127.4, CE = 40. The limit of detection for voriconazole was 5 pg.

2.4. Blood and tissue processing and testing

Following euthanasia and cardiac puncture for HD, LD, C, and NT groups, whole blood was collected into lavender-topped tubes, inverted 10 times and stored under refrigeration, or into red-topped tubes, coagulated, and centrifuged followed by serum extraction into clean red-topped tubes and stored at 4 °C. The complete blood count with differential (CBC w/dif.) and serum chemistry panels were performed within 24 h from sample collection by IDEXX Laboratories, Inc. (Westbrook, ME). Serum chemistry samples had the following individual tests performed per sample: alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total bilirubin, total protein, globulin, blood urea nitrogen (BUN), creatinine, cholesterol, glucose, calcium, phosphorous, chloride, potassium, and sodium. The CBC w/diff test included analysis of the following parameters: white blood cell count (WBC), red blood cell count (RBC), hemoglobin (Hgb), hematocrit (Hct), and platelet count. Plasma samples were taken by collecting whole blood into heparinized tubes on day 3 with excess whole blood being collected into heparinized vials on days 14 and 21.

Following thoracotomy, lungs, liver, kidneys, and spleen were removed from each animal. Prior to preserving in formalin, the lungs were inflated with 10% buffered formalin and tied shut with silk suture, the liver was dissected into three parts, and the kidneys were longitudinally bisected. Following processing, tissues were preserved separately in 40–50 mL of 10% buffered formalin for at least 96 h prior to fixing and sectioning. The post-dissection processing steps ensured penetration of the preservative into the tissues. Pathologic evaluation was independently performed in a blinded manner by both an IDEXX pathologist and a pulmonary pathologist.

2.5. Quantitative analysis of lung tissue

Lung tissue slides were digitally scanned at 20× magnification using a ScanScope® CS from Aperio Technologies, Inc. (Vista, CA), and analyzed using ImageScope software, Aperio Technologies, Inc. (Vista, CA). The Cimolai histopathologic inflammatory score was used by a blinded pulmonary pathologist to assess signs of inflammation in lung airways (Cimolai et al., 1992). In this scoring scheme, the histologic response in the lung was graded based on the degree and location of inflammation with possible scores ranging from 0 to 26, least to most severe immunologic response. A second quantitative pathological evaluation of lung tissues was developed through assessment of a novel marker of immune response in the lungs, the Respiratory Bronchiole Index (RBI). Inhalation injury occurs at the respiratory bronchiole-alveolar interface due to inhalation of particulate and solubilized irritants (Pinkerton et al., 1992; Schlage et al., 1998). Therefore, 10 randomly chosen respiratory ducts, the site of transition from the respiratory bronchiole to the alveolar space, were marked on the digitized images of all lung sections where two blinded investigators independently counted the number of pulmonary macrophages present at respiratory bronchiole alveolar ducts sites per slide under 20× magnification. The individual scores were then normalized and averaged between evaluators to provide the RBI. Validation of the RBI was not performed against tissues with known or controlled alveolar macrophage responses, but values were instead used to statistically compare lung tissue between treatment groups.

2.6. Statistical analysis

Statistical analysis of results was performed using JMP®-7 from e-academy Inc. (Ottawa, Ontario, Canada). Results from blood work were evaluated by analysis of variance (ANOVA) with Dunnett's Control test for the NT group with a p-value ≤ 0.05 for significance. RBI scores were also compared between treatment groups using JMP®-7 by ANOVA with a p-value of ≤ 0.05 for significance.

3. Results

3.1. Pharmacokinetics

Peak plasma samples were collected 30 min after completion of nebulization due to previous pharmacokinetic findings in mice (Tolman et al., 2009a). Following BID dosing for 3 days, peak voriconazole concentrations in lung tissue were 0.85 ± 0.63 μg/g wet lung weight for the high dose group and 0.37 ± 0.01 μg/g wet lung weight for the low dose group with corresponding peak plasma concentrations of 0.58 ± 0.30 and 0.09 ± 0.06 μg/mL, respectively (Table 1). Trough voriconazole concentrations taken on day 3 immediately prior to the 08:00 dose were likewise 0.042 ± 0.002 and 0.044 ± 0.004 μg/g wet lung weight in lung tissue for high and low dose groups and 0.01 ± 0.004 and 0.010 ± 0.003 μg/mL in corresponding plasma samples. After additional days of dosing, trough plasma samples were 0.02 and 0.03 μg/mL for high and low dose groups on day 14 and remained consistent with day 21 trough concentrations of 0.03 ± 0.01 and 0.03 ± 0.003 μg/mL for high and low dose groups.

3.2. Serum chemistries and complete blood counts

When all laboratory values were analyzed by treatment groups, statistically significant increases were observed for components of the hepatic function test compared to the NT group, including ALP, albumin, and total bilirubin (Table 2A–D). However, these increases were observed for both inhaled normal saline control and active

Table 1

Day	Group	Voriconazole peak		Voriconazole trough	
		Lung (μg/g)	Plasma (μg/ml)	Lung (μg/g)	Plasma (μg/ml)
3	High dose	0.85 ± 0.63	0.58 ± 0.30	0.042 ± 0.002	0.011 ± 0.004
	Low dose	0.38 ± 0.01	0.09 ± 0.06	0.040 ± 0.004	0.010 ± 0.003
14	High dose				0.023[a]
	Low dose				0.029[a]
21	High dose				0.030 ± 0.011
	Low dose				0.027 ± 0.003

Animals were exposed to 20 min aerosolizations of normal saline (control group) or aerosolized aqueous solutions of voriconazole at 15.625 mg (low dose group) or 31.25 mg (high dose group). Peak samples were collected 30 min after the completion of nebulizations and trough samples were collected immediately before the next scheduled dose. Values are the mean ± standard deviation.

[a] Value represents the observed concentration ($N = 1$).

treatment groups and would not be deemed clinically significant. Similar patterns of statistical significance but clinical irrelevance were found for decreases in gamma globulin, BUN, phosphorous, and WBC as well as with elevations in calcium, phosphorous, sodium, chloride, and platelet count. Sub-group evaluation by day of analysis and animal gender provided similar patterns of sporadic statistical but not clinical significance with the absence of trends, except for consistent decreases in RBC count and corresponding drops in Hct from day 21 to day 28 for C, LD, and HD treatment groups (data not shown).

Table 2

(A)

Hepatic function	NT	C	LD	HD
ALP (U/L)	188 ± 40	216* ± 52	218* ± 51	213* ± 41
ALT (U/L)	80 ± 54	79 ± 53	96 ± 78	72 ± 31
AST (U/L)	147 ± 123	132 ± 130	164 ± 160	110 ± 58
Albumin (g/dL)	3.2 ± 0.1	3.3* ± 0.2	3.3* ± 0.2	3.3* ± 0.2
Bilirubin (mg/dL)	0.1 ± 0	0.1* ± 0	0.1* ± 0	0.1* ± 0

(B)

Renal function	NT	C	LD	HD
Protein (g/dL)	6.2 ± 0.3	6.1 ± 0.4	6.2 ± 0.3	6.2 ± 0.4
Globulin (g/dL)	3.1 ± 0.3	2.8* ± 0.3	2.9* ± 0.3	2.9* ± 0.3
BUN (mg/dL)	21 ± 2	19* ± 2	21 ± 5	21 ± 3
Creatinine (mg/dL)	0.5 ± 0	0.5 ± 0.1	0.5 ± 0.1	0.4* ± 0.1

(C)

Blood chemistry	NT	C	LD	HD
Cholesterol (mg/dL)	94 ± 10	95 ± 15	95 ± 13	93 ± 19
Glucose (mg/dL)	319 ± 101	329 ± 143	334 ± 154	291 ± 116
Calcium (mg/dL)	11.2 ± 0.5	11.5* ± 0.6	11.4 ± 0.6	11.3 ± 0.6
Phosphorous (mg/dL)	9.0 ± 1.3	9.6 ± 2.1	8.9 ± 1.9	8.0* ± 1.3
Chloride (mg/dL)	99 ± 2	100 ± 2	101* ± 2	101* ± 2
Potassium (mg/dL)	6 ± 1	6 ± 1	7 ± 2	6 ± 1
Sodium (mg/dL)	142 ± 2	144* ± 2	144* ± 3	145* ± 2

(D)

Hematologic parameters	NT	C	LD	HD
WBC ($10^3/\mu L$)	5.3 ± 2.3	4.1* ± 1.6	4.7 ± 2	5.2 ± 1.9
RBC ($10^6/\mu L$)	8.2 ± 1.3	8.0 ± 0.8	7.9 ± 0.8	8.0 ± 0.7
HGB (g/dL)	15.8 ± 2.3	14.7* ± 1.5	14.7* ± 1.2	15.0 ± 1.0
HCT (%)	50.2 ± 7.2	49.5 ± 5.1	49.1 ± 5.3	48.2 ± 7.6
Platelet ($10^3/\mu L$)	657 ± 240	767 ± 235	685 ± 264	842* ± 182

Animals were exposed to 20 min aerosolizations of normal saline (control group) or aerosolized aqueous solutions of voriconazole at 15.625 mg (low dose group) or 31.25 mg (high dose group). Animals in the no treatment group received no aerosolizations but were housed and cared for according to standard care guidelines. NT = no treatment group ($n = 20$), C = inhaled normal saline group ($n = 30$), LD = inhaled low dose voriconazole group ($n = 30$), HD = inhaled high dose voriconazole group ($n = 30$).

Values are the mean ± standard deviation.

*Statistically significant compared to NT ($p < 0.05$).

3.3. Histopathology

Qualitative pathological descriptions of liver, kidney, and spleen sections suggested that inhalation of high or low dose voriconazole produced no histological differences as compared to inhaled normal saline control (data not shown). However, pathological descriptions of lung tissue suggested inhaled voriconazole promoted an increase in alveolar and respiratory duct macrophages. However, no inflammatory cell infiltrate of neutrophils, eosinophils or lymphoctyes were noted. The lungs did not show pathological changes indicative of ulceration of the airway, interstitial changes, or edema (Fig. 1). Application of the Cimolai scoring system on lung tissues failed to differentiate between treatment groups with scores of 0 to 1, on a 0 to 26 scale. Therefore, quantitative analysis of lung histiocytosis was performed through use of the RBI, which demonstrated significant elevations for both LD and HD groups compared to C group animals for days 7, 14, 21, and 28 (Fig. 2). In addition, RBI values between LD and HD were not statistically different ($p > 0.05$).

4. Discussion

The poor prognosis of IPA has prompted investigation of targeted antifungal delivery to the lungs via nebulization. Positive results following initial animal experimentation with inhaled amphotericin B led investigators to early clinical trials with varying results (Beyer et al., 1994; Schmitt et al., 1988; Dubois et al., 1995; Erjavec et al., 1997). Animal studies also suggested positive clinical outcomes could exist for aerosolized suspension of nano-structured itraconazole (Alvarez et al., 2007; Hoeben et al., 2006; Vaughn et al., 2007). Both aerosolized amphotericin B and itraconazole reported high lung tissue concentrations with very low systemic drug distribution following inhalation. In one study, high tissue concentrations but low systemic drug distribution following targeted antifungal delivery was supplemented with systemic administration of the antifungal and suggested improved survival as treatment in a rodent model of IPA (Gavalda et al., 2005). The contributory effect of an inhaled and targeted antifungal with systemic drug distribution could improve survival through prevention of fungal dissemination from alveolar spaces into the pulmonary capillaries as observed following inhalation only (Ruijgrok et al., 2001).

The pharmacokinetic properties and dose tolerability of inhaled aqueous solutions of voriconazole were dependant on the dose that was deposited in the lungs that was then available for systemic absorption and distribution. Although a deposited dose of voriconazole was not quantified in this study, the aerodynamic properties of the aerosol have been reported and suggest a large fraction of the inhaled dose was available for absorption from the

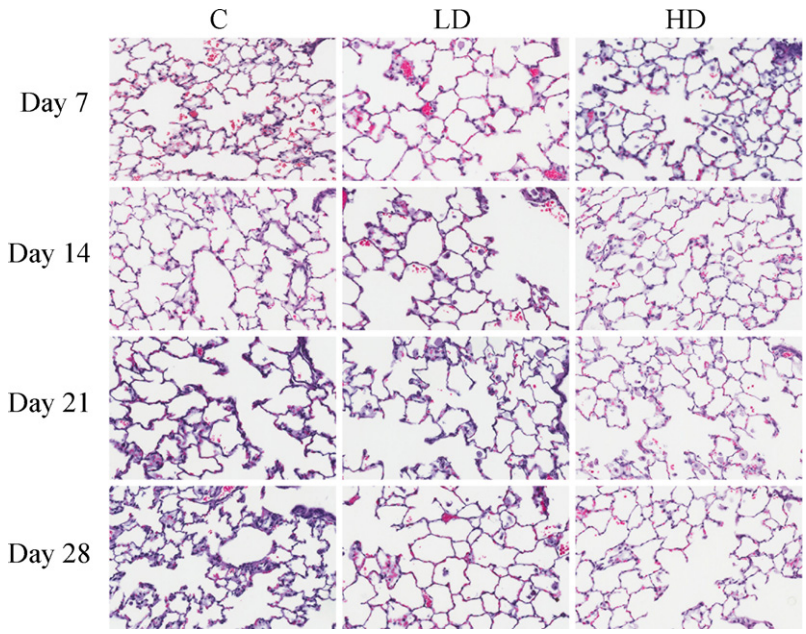

Fig. 1. Representative histopathology images following inhalation of voriconazole. C = control group, LD = low dose group, HD = high dose group. Representative images are of alveolar regions near the respiratory bronchiole duct. Free alveolar macrophages are visible in several images and demonstrate the variability observed between treatment groups as well as the duration of treatment or recovery.

lung tissue. Specifically, Tolman and colleagues reported the aerodynamic properties, including the total emitted dose, fine particle fraction, mass median aerodynamic diameter, and geometric standard deviation, were 25.51 ± 6.25 mg, 71.7 ± 2.62%, 2.98 ± 0.06 μm, and 2.20 ± 0.13, respectively (Tolman et al., 2009a).

Correlations have been suggested between plasma drug concentrations and adverse events as well as efficacy against IPA for voriconazole in humans (Pascual et al., 2007; Denning et al., 2002). However, substantial intra-patient as well as intra-species variability in absorption, distribution, and metabolism associated with oral and intravenous voriconazole limit the utility of human-defined peak or trough voriconazole concentrations associated with efficacy and toxicity with the pharmacokinetic findings in this study (Davis et al., 2006; Brueggemann et al., 2008; Roffey et al., 2003; Capitano et al., 2006). Thus, the dose of inhaled voriconazole used in the HD group, equivalent to a 31.25 mg exposure dose, was efficacious in an immunosuppressed murine model of IPA with 67% survival over 12 days. Although voriconazole concentrations were not assessed in infected animals, peak and trough plasma concentrations following multiple doses in healthy mice were 2.32 and 0.28 μg/mL, respectively, that were lower than those associated with efficacy in humans (Pascual et al., 2008). These same healthy mice had peak lung concentrations of 6.73 μg/g wet lung weight following multiple doses which likely contributed to improved murine survival (Tolman et al., 2009b,a).

In the present study, peak voriconazole concentrations were variable with a wide standard deviation indicative of variability in animal studies and influenced to a greater manner by slight differences in absorption and distribution processes following inhalation. Trough voriconazole concentrations were much more uniform with a narrow standard deviation and suggest uniformity in drug elimination processes in this animal model of drug inhalation. In addition, HD group rats had an equivalent peak tissue to plasma concentration ratio, 1.5 to 1, following 3 days of BID dosing while a higher ratio was observed in LD group rats, 4.1 to 1, with corresponding trough concentration ratios of 3.8 to 1 and 4.0 to 1. These varied partition factors based on concentration ratios between tissue and plasma are likely due to altered voriconazole metabolism as demonstrated by induction of voriconazole metabolism following oral and IV doses in rodents (Roffey et al., 2003; Sugar and Liu, 2000). Nevertheless, moderate trough plasma voriconazole accumulation was observed following BID dosing for up to 21 days, necessitating further pharmacokinetic analysis of voriconazole in lung tissue and plasma following inhalation to verify metabolic induction-based pharmacokinetic variations.

Despite possible changes in voriconazole pharmacokinetics following multiple inhalations in a rodent model, drug distribution to the plasma was markedly improved compared to reports of drug distribution following inhalation of amphotericin B and itraconazole in the literature (Lowry et al., 2007; Marra et al., 2002; Hoeben et al., 2006; Vaughn et al., 2006; Yang et al., 2008b). The relative elevation of voriconazole plasma drug concentrations were likely a result of improved drug solubility due to the cyclodextrin in the formulation leading to improved drug absorption in the lungs (Nakate et al., 2003; Miyake et al., 1999; Carrier et al., 2007; Irie and Uekama, 1997). Although the toxicological profile of inhaled sulfobutyl ether-

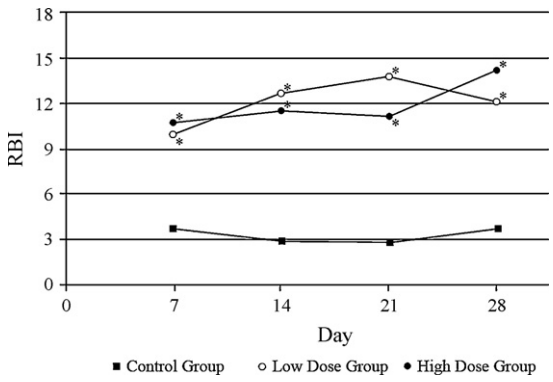

Fig. 2. Animals were exposed to 20 min aerosolizations of normal saline (control group) or aerosolized aqueous solutions of voriconazole at 15.625 mg (low dose group) or 31.25 mg (high dose group). RBI = respiratory bronchiole index. *Statistically significant difference compared to Control Group ($p < 0.05$). Error bars omitted due for clarity in interpreting the figure. However, the average values ± standard deviation for days 7, 14, 21, and 28 were: 3.8 ± 3.3, 2.9 ± 2.7, 2.8 ± 2.5, and 3.8 ± 3.8 for the control group; 10.1 ± 4.8, 12.9 ± 7.3, 13.9 ± 8.2, and 12.2 ± 10.6 for the low dose group; and 11.0 ± 6.2, 11.7 ± 4.2, 12.8 ± 8.9, and 14.4 ± 7.9 for the high dose group.

β-cyclodextrin has not been publicly disclosed, limited data with similar cyclodextrin agents have been shown to be safe and well tolerated following inhalation (Stella and He, 2008; Evrard et al., 2004). Specifically, Evrard et al. demonstrated inhaled cyclodextrins were well tolerated through evaluation of pulmonary epithelium for histological signs of inflammation, morphology and cellular distribution of histiocytes in bronchoalveolar lavage fluid (BAL), bronchial hyperresponsiveness, kidney histology, and BUN values. Thus, the differences between treatment and control groups likely represent responses to inhaled voriconazole and not the cyclodextrin.

Elevated hepatic function values (Table 2A) were observed in all treatment groups and represent artifacts resulting from the method of euthanasia and duration of hypoxia prior to cardiac puncture and blood sampling (Hursh et al., 1987; Gelman, 1986). In addition, observed hyperglycemia (Table 2C), not statistically significant between NT and treatment groups (HD, LD, and C groups), may be linked to stress imposed through chronic handling and manipulation during animal dosing as well as the use of isoflurane in euthanasia (Lattermann et al., 2001; Zardooz et al., 2006; Saha et al., 2005). Numerous laboratory test values were statistically significant between NT and treatment groups but without clinical significance either due to comparable differences between C and LD or HD groups; e.g. ALP, albumin, bilirubin, globulin, sodium (Table 2A–C); or isolated differences in one group without evidence of trends between groups or throughout treatment; e.g. BUN, creatinine, calcium, phosphorous, WBC, Hgb, and platelet count (Table 2B–D).

Qualitative pathological evaluation of liver, kidney, and spleen tissue samples demonstrated no difference between treatment groups as well as no differences for sub-group analysis based on sample day or gender between HD or LD groups and the control group. Pathologist descriptions of lung tissues, however, suggested inhaled voriconazole high dose and low dose groups could have increased frequency of histiocytes in the respiratory ducts compared to the inhaled normal saline control group. However, no histologic changes in the airway or interstitial space were noted. Additionally, no inflammatory response (e.g. migration of neutrophils, eosinophils, or lymphocytes) was seen at any time point in either the C or HD and LD groups. Thus, pathologic description of the lungs following inhaled voriconazole suggest isolated elevations in alveolar macrophages that would resolve following cessation of therapy (Fels and Cohn, 1986; Yang et al., 2008a).

Quantitative evaluation of lung tissue inflammation through the Cimolai scoring system, as previously utilized (Vaughn et al., 2007), was not able to differentiate between treatment groups with values of 0 for all groups. Specifically, voriconazole treated lungs had no signs of airway inflammation, peri-vascular inflammation, or signs of pneumonia that are weighted heavily in the Cimolai Score. Therefore, the RBI, was developed to assess the presence of observed alveolar macrophages at some respiratory bronchiole-alveolar spaces. Alveolar macrophages, a non-specific first-line host defense response to inhaled foreign molecules at the respiratory duct, represented an objective comparison of histiocyte presence at the most-likely site for deep-lung tissue damage or irritation. Alveolar macrophages were grossly typical in morphology with granulated or vesiculated cells, indicative of phagocytic processes, present in some tissues. Significant elevations in the RBI were observed for HD and LD groups compared to the control group for all days tested (Fig. 2). No significant difference was observed between HD and LD groups. However, substantial inter- and intra-animal variability was observed in the RBI and could be due to regional differences in drug deposition following inhalation within the lung (Fig. 1). Although not employed previously as a quantitative measure, the RBI should return to baseline levels after a longer recovery period due to a delay in alveolar macrophage elimination kinetics following a stimuli (Brazil et al., 2005). Additional studies are needed to document the reversibility of RBI elevations after cessation of inhaled voriconazole.

5. Conclusions

Inhaled aqueous solutions of inhaled voriconazole were well tolerated following multiple doses in rats. The pharmacokinetic profile in rats following multiple inhalations demonstrated good drug absorption into the systemic circulation from the lungs due to the sulfobutyl ether-β-cyclodextrin present in the formulation. Some abnormal laboratory test values were statistically significant but did not correlate with clinically importance with hepatotoxicity and hyperglycemia being artifacts associated with methodological procedures in the study. Pathological evaluation of liver, kidney, and spleen tissues demonstrated good inhaled drug tolerability but with acute elevations in alveolar macrophages present at the respiratory duct associated with inhaled voriconazole. Additional studies are needed to further characterize pharmacokinetic and laboratory test parameters and lung histopathology changes before utilization in patient populations.

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