Isavuconazole (BAL4815) Pharmacodynamic Target Determination in an \textit{in vivo} Murine Model of Invasive Pulmonary Aspergillosis against Wild Type and \textit{Cyp51} Mutant Isolates of \textit{Aspergillus fumigatus}

Running Title: Isavuconazole Aspergillus Pharmacodynamics

Authors: Alexander J. Lepak\textsuperscript{a}, Karen Marchillo\textsuperscript{a}, Jamie vanHecker\textsuperscript{a}, David R. Andes\textsuperscript{a#}

Author’s Affiliation:
\textsuperscript{a}University of Wisconsin, Madison, Wisconsin, USA

\textsuperscript{#}Corresponding Author:
David R. Andes, MD
Department of Medicine
Department of Medical Microbiology and Immunology
University of Wisconsin
1685 Highland Ave, MFCB, Room 5211
Madison, WI 53705-2281
dra@medicine.wisc.edu
ABSTRACT

Invasive pulmonary aspergillosis (IPA) continues to rise in concert with increasing numbers of immune suppression techniques to treat other medical conditions and transplantation. Despite these advances, morbidity and mortality remain unacceptably high. One strategy utilized to optimize outcomes is antifungal pharmacodynamic (PD) examination. We explored the pharmacodynamics of a new triazole in development, isavuconazole, in a murine neutropenic IPA model. Ten A. fumigatus isolates were used including 4 Cyp51 wild type isolates and 6 Cyp51 mutants. MIC range was 0.125 - 8 mg/L. Following infection groups of mice were treated with 40 - 640 mg/kg/12 h oral prodrug (BAL8557) for 7 days. Efficacy was determined by quantitative PCR of lung homogenates. At the start of therapy mice had 4.97 log_{10} conidial equivalents (C.E.)/ml of lung homogenate and increased to 6.82 log_{10} C.E./ml of lung homogenate in untreated animals. The infection model was uniformly lethal in untreated control mice. PD target endpoints examined included static dose AUC/MIC and 1 log_{10} kill AUC/MIC. A stasis endpoint was achieved for all isolates with an MIC of ≤ 1 mg/L and 1 log_{10} kill in all isolates with an MIC of ≤ 0.5 mg/L, regardless of presence or absence of Cyp51 mutation. The static dose range was 65 - 617 mg/kg/12 h. The corresponding median free drug AUC/MIC was near 5. The 1 log_{10} kill dose range was 147 - 455 mg/kg/12 h and corresponding median free drug AUC/MIC of 11.1. These values are similar to those previously reported with other triazoles.
INTRODUCTION

Invasive pulmonary aspergillosis (IPA) is a common cause of morbidity and mortality in immunocompromised patients (1-7). The development of *Aspergillus* active triazoles was a major step forward in therapy; however, morbidity and mortality remain unacceptably high. Additionally, the emergence of Cyp51 mutant isolates that confer decreased susceptibility to triazoles is a threat to the efficacy of this drug class (8-13). Therefore, development of novel compounds and examination of the pharmacodynamic relationships of drug exposure, MIC, and outcome are necessary for the optimal utilization of these drugs.

Pharmacodynamic studies integrate the pharmacokinetic properties of a drug, *in vitro* potency (MIC), and treatment efficacy. Common goals of these PD studies are to maximize clinical outcome through dosing optimization and assist in susceptibility breakpoint determination. These investigations have been integral in the optimal use of antibiotics for bacterial infections and antifungal agents for mucosal and invasive candidiasis (14-18). However, only recently have pharmacodynamic investigations been utilized for filamentous fungal infections such as IPA (19-23).

Isavuconazonium sulfate (BAL8557) is the water-soluble prodrug of isavuconazole (BAL4815), a novel triazole compound, with potent activity against numerous fungal pathogens including *Aspergillus* species (24-28). After intravenous or oral administration the prodrug is rapidly cleaved by plasma esterases to form the active drug isavuconazole (BAL4815) and an inactive cleavage product BAL8728 (25, 29, 30).

The drug is currently in clinical development, including two phase III trials examining its efficacy for patients with IPA ([http://clinicaltrials.gov](http://clinicaltrials.gov), NCT00634049 & NCT00412893). The pharmacodynamic relationships of isavuconazole have been examined in experimental models of invasive candidiasis, however, pharmacodynamic evaluation and targets are unknown for IPA. The objectives of the current study were to [1]...
examine the pharmacodynamic relationship of isavuconazole in a murine model of IPA and [2] define the optimal isavuconazole exposure for infection due to both wild type and Cyp51 mutant isolates.

**MATERIALS AND METHODS**

**Organisms.** Ten *Aspergillus fumigatus* isolates were chosen including 9 clinical isolates with and without Cyp51 mutations and one laboratory isolate with an Fks1 mutation. Organisms were grown and subcultured on Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, MI). The organisms were chosen based on similar fitness as determined by growth in lungs and mortality in untreated animals.

**Drug.** Prodrug isavuconazonium sulfate (BAL8557) and isavuconazole (BAL4815) powder were provided by the sponsor (Astellas) for in vivo and in vitro studies, respectively. The prodrug was dissolved in sterile water and buffered to pH of 4.0 prior to oral administration. Isavuconazole powder was dissolved in DMSO per sponsor instructions prior to in vitro susceptibility testing. All dosages in the study were administered by oral route and based upon oral prodrug dose. All concentration measurements (i.e. PK data and calculated PK) are based upon active drug. A conversion factor is necessary to compare equivalent prodrug and active drug on a mg/kg basis. This conversion factor was determined based on a prodrug:drug equivalency rating of 1.863 (provided by the sponsor) and 89% purity of the prodrug powder. Thus, the conversion factor for determining equivalent isavuconazole dose from prodrug dose was 0.48 (i.e. for every 1 mg/kg of prodrug administered orally, the equivalent in vivo isavuconazole dose would be 0.48 mg/kg). The purity of isavuconazole powder for in vitro susceptibility testing was >99%.
**In vitro susceptibility testing.** All isolates were tested by broth microdilution in accordance with CLSI document M38-A2 (31). MICs were performed in duplicate three times. The median value is reported in Table 1.

**Animals.** Six-week old Swiss/ICR specific-pathogen-free female mice weighing 23 - 27 g were used for all experiments (Harlan Sprague-Dawley, Indianapolis, IN). Animals were housed in groups of five and allowed access to food and water *ad libitum*. Animals were maintained in accordance with the American Association for Accreditation of Laboratory Care criteria (32). Moribund animals showing distress were sacrificed prior to study endpoint (7 days) in accordance with the humane treatment of laboratory animals. The animal research committee of the William S. Middleton Memorial VA Hospital and University of Wisconsin-Madison approved the animal studies.

**Infection Model.** Mice were rendered neutropenic (polymorphonuclear cells <100/mm$^3$) by injection of 150 mg/kg cyclophosphamide subcutaneously (SC) on days -4 and -1. Prior studies have shown this maintains neutropenia for 4 days; therefore, an additional injection (150 mg/kg) was administered on day +3 to ensure neutropenia throughout the entire 7-day experiment (20, 33, 34). Additionally, cortisone acetate 250 mg/kg SC was administered on day -1 as previously described (20). Throughout the 7-day experiment mice were also given ceftazidime 50 mg/kg/d SC to prevent opportunistic bacterial infection. We have previously shown uninfected control animals given the above immune suppression and antibiotic prophylaxis have 100% survival to study endpoint, whereas infected controls that were untreated have 100% mortality prior to study endpoint (20).

Organisms were subcultured on PDA 5 days prior to infection and incubated at 37° C. On the day of infection, the inoculum was prepared by flooding the culture plate
with 5 ml of normal saline and 0.05% Tween-20. Gentle agitation was applied to release the conidia. The conidial suspension was collected and quantified by hemocytometer (Bright-Line, Hausser Scientific, Horsham, PA). The suspension was diluted to a final concentration of 1.2 x 10^7 conidia/ml. Viability was confirmed by plating the suspension and performing CFU counts.

An aspiration pneumonia model was utilized as previously described (20). Briefly, mice were anesthetized with a combination of ketamine and xylazine. Fifty microliters of the 1-2 x 10^7 conidia/ml suspension was pipetted into the anterior nares with the mice held upright to allow for aspiration into the lungs. As previously shown and confirmed in the current study (data not shown) this results in invasive aspergillosis in over 90% of animals and 100% mortality in untreated infected mice prior to the study endpoint (20). Drug treatment commenced two hours after initiation of infection.

**Lung processing and organism quantitation.** Processing and quantitation of *Aspergillus* burden in the lungs of mice was performed as previously described (35, 36). Briefly, at the time of sacrifice for moribund animals or at the end of therapy (7 days), lungs were aseptically harvested and placed in a 2-ounce sterile polyethylene Whirl-Pak bag (Nasco, Fort Atkinson, WI) containing 2 ml of sterile 0.85% NaCl. The lungs were manually homogenized using direct pressure (37) to produce a primary homogenate. One ml of this primary homogenate was then placed in a sterile bead beating tube (Sarstedt, Newton, NC) with 700 μl of 425-600 μm acid-washed glass beads (Sigma-Aldrich, St. Louis, MO). The primary homogenate was bead-beaten in a Bio-spec bead beater (Bartlesville, OK) for 90 s at 4200 rpm to yield a secondary homogenate. One hundred μl of this secondary homogenate was mixed with 100 ul Buffer ATL (Qiagen, Valencia, CA) and 20 μl proteinase K (Qiagen, Valencia, CA) and incubated overnight at 56° C with gentle agitation. DNA was then isolated following the DNEasy Blood and
Tissue protocol (Qiagen, Valencia, CA). A final DNA elution step was performed with 100 μl elution buffer (Qiagen, Valencia, CA). The DNA was stored at -20°C until the day of PCR.

Quantitative PCR (qPCR) plates were prepared on the day of assay. Standard quantities of conidia were prepared by hemacytometer counts and were utilized to generate standard curves. The results are reported as conidial equivalents (C.E.) per ml of lung homogenate. Samples were assayed in triplicate using a Bio-Rad CFX96 Real-time system (Hercules, CA). A single copy gene, Fks1, was chosen for quantitation (38).

Primer sequences included: forward primer 5'-GCCTGGTAGTGAAGCTGAGCGT-3', reverse primer 5'-CGGTGAATGTAGGCATGTTGTCC-3', and probe 5'-6-FAM-AGCCAGCGCCGCAAATG-MGB-3' (Integrated DNA Technologies, Coralville, IA).

The Fks1 mutation (EMFR S678P) was not located in the primer:probe area of the genome. All isolates have been previously studied in a spiking experiment of known quantities of conidia into lung homogenate to ensure the primer:probe set performs similarly for all isolates over the dynamic range (10^2 - 10^8) (20).

Pharmacokinetics. Single dose pharmacokinetics of isavuconazole (BAL4815) were determined in individual ICR/Swiss mice following oral administration of the prodrug (BAL8557) at 10, 40, 160, and 640 mg/kg in 0.2 ml volumes by oral-gastric gavage (OG). Plasma from groups of three isoflurane-anesthetized mice were collected at each of 7 time points (0.5, 1, 2, 4, 8, 12, and 24 h). The plasma was stored at -80°C until day of drug assay measurement. Drug concentration measurements were performed by the sponsor using LC-MS as previously described.

A non-compartmental model was used in the PK analysis. Pharmacokinetic parameters including elimination half-life and concentration at time zero (C_0) were calculated via nonlinear least-squares techniques. The AUC was calculated by the
trapezoidal rule. For treatment doses in which kinetics were not directly determined, pharmacokinetic parameters were estimated by linear interpolation for those doses between two measured doses and by linear extrapolation for doses above or below the highest and lowest measured doses. Protein binding (99%) was based on previous studies in mice by the sponsor (personal communication).

Pharmacodynamic index and magnitude. The AUC/MIC was used as the PD index for exploration of exposure response relationships based upon previous PK/PD investigations with triazoles (19-22). Both total and free (non-protein bound) drug concentrations were considered. Neutropenic mice were infected as described above. Treatment consisted of 2-fold increases in prodrug (BAL8557) concentration (range 40 - 640 mg/kg) administered q12 h by OG gavage for 7 days. The doses were selected to vary effect from maximal to no efficacy and included exposures expected with regimens under study in clinical trials. Controls were utilized for each isolate and included a zero hour and untreated controls. Four mice were included in each treatment and control group.

Data analysis. The qPCR data was modeled according to a Hill-type dose response equation: \( \log_{10} D = \log_{10} \left( \frac{E - E_{\text{max}}}{N} + \log_{10} \text{ED}_{50} \right) \), where \( D \) is the drug dose, \( E \) is the growth over the study period as measured by qPCR and represented as C.E./ml lung homogenate in untreated control mice, \( E_{\text{max}} \) is the maximal drug effect, \( N \) is the slope of the dose-response curve, and \( \text{ED}_{50} \) is the dose needed to achieve 50% maximal effect. The AUC/MIC for total and free drug was determined for each isolate. The coefficient of determination \( (R^2) \) was used to estimate the percent variance in the change in \( \log_{10} \) C.E./ml of lung homogenate over the treatment period for the different dosing regimens that could be attributed to the PD index (AUC/MIC). The dose necessary for net stasis
(static dose, SD) and 1-log kill were determined when these endpoints were achieved. Additionally, the PD target total and free drug AUC/MIC associated with these endpoints was calculated. The SD and PD target total and free drug AUC/MIC were compared between wild-type and Cyp51 mutant isolates by t-test for normally distributed data and by Mann-Whitney rank sum test for non-normally distributed data.

RESULTS

Organism susceptibility and in vivo fitness. Isavuconazole (BAL4815) susceptibility testing, genotype, and relative fitness in the in vivo murine model of each isolate are shown in Table 1. Cyp51 wild type MICs ranged from 0.25 - 1 mg/L and from 0.125 - 8 mg/L in Cyp51 mutants. The organisms exhibited similar in vivo fitness based on increase in burden in untreated animals until the time of death or sacrifice. At the start of therapy mice had 4.97 ± 0.33 log_{10} C.E./ml lung homogenate and the burden increased to 6.82 ± 0.51 log_{10} C.E./ml lung homogenate in untreated animals. Each isolate produced 100% mortality prior to study endpoint in untreated animals with death occurring between days 3 and 6 in all untreated animals.

Pharmacokinetics. The time courses for isavuconazole (BAL4815) in the plasma of mice following OG doses of 640, 160, 40, and 10 mg/kg of prodrug (BAL8557) are shown in Figure 1. Peak levels were achieved within 2 h for each dosing regimen and ranged from 0.51 to 25.4 mg/L. The elimination half-life in serum increased in a dose-dependent fashion from 1 to 5 h. The AUC from 0 h to infinity (AUC_{0-∞}), as determined by the trapezoidal rule, ranged from 0.9 to 287 mg*h/L. The AUC was relatively linear over the dose range (R^2 0.98).
Dose-response curves. A dose-response relationship was observed for each isolate with higher doses of isavuconazole achieving a larger microbiologic effect (Figure 2). However, higher doses were necessary to achieve similar microbiologic effect against isolates with elevated isavuconazole MICs. A net static outcome and cidal activity was achieved for all wild type isolates as well as 2 of 6 Cyp51 mutants. Maximal effect against wild type isolates was approximately a 2 log_{10} reduction in organism burden compared to that in the lungs at the start of therapy and nearly a 4 log_{10} compared to untreated controls at the end of therapy.

PD index and target magnitude. The doses needed to produce net growth suppression (i.e. static dose) and to produce 1 log_{10} kill are shown in Table 2. Net stasis was achieved in all isolates that exhibited an MIC of ≤1 mg/L; whereas 1 log_{10} kill was observed in all isolates with an MIC of ≤0.5 mg/L. Therefore, we were able to estimate the static dose AUC/MIC target for all 4 Cyp51 wild type isolates and 2 of 6 Cyp51 mutants, these latter two (F14403 and F14532) exhibiting lower MIC values than other four mutant isolates. The static dose for Cyp51 wild type isolates ranged from 212 – 617 mg/kg/12 h. In comparison, the static dose for the two mutant isolates, F14403 and F14532, were 65 and 515 mg/kg/12 h, respectively. The 1 log_{10} kill PD target was achieved in 3 of 4 Cyp51 wild type isolates and only for a single mutant isolate. The 1 log_{10} kill dose for Cyp51 wild type isolates ranged from 302 – 455 mg/kg/12 h; whereas it was 147 mg/kg/12 h for the single mutant isolate (F14403) for which this endpoint was achieved.

Total and free drug AUC/MIC PD targets are shown in Table 2. The free drug AUC/MIC associated with net stasis for the Cyp51 wild type group ranged from 4.15 – 11.1; whereas for the two mutant isolates for which this endpoint was slightly lower at 3.61 – 3.67. The difference between the two groups was not statistically significant (p =
For all isolates where net stasis was achieved the median static dose free drug AUC/MIC was 5.0. The 1 log₁₀ kill free drug AUC/MIC was roughly 2-fold higher than the static dose PD target, with a median value of 11.1. The AUC/MIC values and treatment outcome for all organisms was fit to the Hill sigmoid dose-response model and the relationship is shown in Figure 3. AUC/MIC was a strong predictor of observed outcome with an $R^2$ of 0.75.

DISCUSSION

Aspergillus active triazoles have become the cornerstone for prevention and treatment of invasive aspergillosis (39). Pre-clinical animal model pharmacokinetic/pharmacodynamics (PK/PD) investigation has proven useful for optimizing therapy for numerous pathogens but has been under-utilized for filamentous fungal pathogens such as Aspergillus. These pharmacodynamics studies are important in the development of novel triazoles, such as isavuconazole, to provide a framework for predicting drug exposures that are expected to achieve a successful therapeutic outcome. Additionally, with the emergence of Aspergillus drug resistance to the triazole class, mediated by mutations in the Cyp51 gene, these studies are integral in examination of susceptibility breakpoints.

PK/PD studies for isavuconazole have been limited to two previous murine disseminated candidiasis models (40, A. J. Lepak, K. Marchillo, J. vanHecker, and D. R. Andes, submitted for publication). Both showed a very strong relationship between the PD index AUC/MIC and treatment outcome. Results from the current study also demonstrated a strong relationship between total dose and effect in a murine invasive pulmonary aspergillosis model. While this is the first PK/PD examination for Aspergillus for the triazole isavuconazole, previous studies using these models with the triazoles, voriconazole and posaconazole, provide the opportunity to comparison across the class.
Employing the same model and diverse group of isolates in the current study, we have previously shown a posaconazole free drug AUC/MIC of approximately 1 was associated with net stasis (20). Similar studies by other investigators using pulmonary model and intravascular models found a posaconazole free drug AUC/MIC of 1.67 was associated with 50% maximal efficacy (ED$_{50}$) and a value of 3.2 was associated with 50% increase in survival, respectively (19, 21). Previous study with voriconazole in a murine pulmonary aspergillosis model observed a 50% maximal effect with a free drug AUC/MIC of 11 (22). The static dose PD target free drug AUC/MIC identified in this study for isavuconazole at a median value of 5.0 is congruent with these other in vivo pharmacodynamic triazole studies. For comparison purposes, the ED$_{50}$, which is shown on Figure 3, was a free drug AUC/MIC of approximately 7.

In current model we utilized qPCR which has previously been shown to provide a large dynamic range between effective and ineffective therapy, reproducibility among biological replicates, and correlates very well with mortality (20). The static dose and 1 log$_{10}$ dose were the primary PD target endpoints used in this study. It is unclear which PD endpoint in the animal model correlates with optimal treatment effect in patients. Further clinical PK/PD studies utilizing large sets of patient data, organism susceptibility, and treatment outcome to delineate the optimal clinical PK/PD target are urgently needed.

In summary, we have shown the isavuconazole PD index AUC/MIC correlates well with treatment outcome in a murine model of IPA. MIC was a strong predictor of success or failure regardless of presence or absence of a Cyp51 mutation. Mutations that lead to elevated MICs to other triazoles did not universally correlate with elevated isavuconazole MIC. The median total and free drug 24 h AUC/MIC PD target for net stasis was 503 and 5, respectively. This target correlates well with other triazole studies
utilizing this model. Further clinical study with isavuconazole for IPA is warranted and it may be a useful addition to the triazole armamentarium for invasive aspergillosis.

ACKNOWLEDGEMENTS

Astellas provided funding for the studies. We kindly thank Dr. David Perlin for providing isolates DPL EC S 1 and EMFR S678P.

Figure 1. Plasma concentrations of isavuconazole (BAL4815) after administration of oral prodrug (BAL8557) at 640, 160, 40, and 10 mg/kg. Each symbol represents the geometric mean ± standard deviation from three mice. The peak concentration (Cmax), 24 h AUC0-∞ (AUC), and elimination half-life (T1/2) are shown for each dose.

Figure 2. In vivo isavuconazole dose-response curves for multiple A. fumigatus isolates. Oral prodrug (BAL8557) was administered in 2-fold increasing concentrations from 40 to 640 mg/kg by OG route every 12 h for a 7 d duration. Open symbols represent Cyp51 wild type isolates and closed symbols Cyp51 mutants. Each symbol represents the geometric mean ± standard deviation of organism burden as measured by qPCR.

Figure 3. Relationship between PD index total drug AUC/MIC and treatment efficacy for isavuconazole against ten A. fumigatus isolates. Open symbols represent Cyp51 wild type isolates and closed symbols Cyp51 mutants. Each data point represents the geometric mean of organism burden in four mice. A
best-fit line based on the Hill equation is included. The PD parameters \( E_{\text{max}} \), \( ED_{50} \), slope (N), and coefficient of determination \( (R^2) \) are shown in the figure legend.

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oral administration of its prodrug, BAL8557, in healthy volunteers. Antimicrobial agents and chemotherapy 50:286-293.


Table 1. *In vitro* susceptibilities and *in vivo* fitness of select *A. fumigatus* isolates.

<table>
<thead>
<tr>
<th><em>A. fumigatus</em> isolate</th>
<th>Isavuconazole (BAL4815) MIC (mg/L)</th>
<th>Posaconazole MIC (mg/L)</th>
<th><em>In vivo</em> fitness&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>0.25</td>
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<sup>a</sup> Defined as the growth, measured in log<sub>10</sub>C.E./ml lung homogenate, of the isolate in untreated animals until time of death or sacrifice.
Table 2. *In vivo* pharmacodynamic efficacy of isavuconazole in an immunocompromised murine pulmonary aspergillosis model.

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<th>Organism</th>
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<th>1 log kill Dose (mg/kg/12 h)</th>
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Blank cells represent strains for which the endpoint was not achieved.