Standard Operating Procedure (SOP)
***Candida auris* Murine Invasive Candidiasis Model**
NIH/NIAID Task Order A01

Isolate:

- *Candida auris* clinical isolate #DI17-46, originally cultured from the bloodstream of a patient with invasive candidiasis, was used in order to establish infection. The species identification was confirmed by DNA sequence analysis of the ITS and D1/D2 domains of rRNA.
- MICs at 24 hrs performed by the CLSI M27-A3 method and results reported in µg/ml [1].
    - Posaconazole 0.25
    - Voriconazole 1
    - Fluconazole >64
    - Itraconazole 1
    - Caspofungin 0.25
    - Micafungin 0.125

Mice:
Outbred male ICR (CD-1) mice have been evaluated and used in this model of invasive candidiasis.

Supplier for this strain that has been used is Envigo (www.envigo.com). The typical weight and age range used is shown in the table below:

Strain	Weight	Age
ICR (CD-1)	26 - 30 grams	4 - 5 weeks

Immunosuppression:
This murine model is performed using neutropenic mice. A single dose of 5-fluorouracil is used to render the mice neutropenic.

- 5-fluorouracil (50 mg/mL vial, NDC#0703-3015-11, Teva Pharmaceutical) is used without further dilutions.
- Administer 0.1 mL (5 mg) dose per mouse intravenously one day prior to inoculation.
- This single dose results in profound and prolonged neutropenia (< 100 neutrophils/mm^3 for > 10 days) [2].

Inoculum Preparation and Quantification:

- Subculture the *C. auris* strain twice at 37°C for 48 hours on Sabouraud dextrose agar prior to in vivo use.
- Place isolates taken from the second subculture into 50 mL of brain heart infusion broth and allow to grow overnight (~18-24 hours) in a shaking incubator at 37°C and 200 rpm.
- Collect the cells by centrifugation (~2,000 rpm for 10 minutes) and wash in sterile

physiologic saline or sterile phosphate buffered saline (PBS) with 0.1% Tween 20. Remove the supernatant and repeat the wash 2 additional times in sterile physiologic saline or sterile PBS with 0.1% Tween 20. After the third wash collect a loopful (10µl) of cells and place into 2 mL of sterile saline or PBS with 0.1% Tween 20. Spin in a microcentrifuge at high speed for 3 minutes. Discard the supernatant and then resuspend the cells in 2 mL of sterile saline or PBS with 0.1% Tween 20.

- Prepare dilutions of the cells in sterile saline or PBS with 0.1% Tween 20 (e.g., 1:200 to 1:400 or 1:1000 to 1: 10,000) and determine the number of cells/mL using a hemocytometer.
    - Adjust the desired infecting inoculum (e.g. 3 x10^7 cells/mL for survival studies or 5 x 10^6 cells/mL for tissue burden studies).
    - The inoculum should be used to infect the mice within 2 hours of preparation.

  NOTE: It is essential to regularly resuspend the suspension during the infection.
- Confirm the inoculum viability by serially diluting an aliquot of the inoculum in sterile saline or phosphate buffered saline with 0.1% Tween 20. Prepare serial dilutions (e.g., 1:1000, 1:10,000, and 1:100,000) of the stock, and plate 100 microliters of the dilutions onto Sabouraud dextrose agar. Incubate the plates at 37°C and count the number of colonies the 48 hours later.

Intravenous Inoculation:

- Inoculate each mouse by injecting 0.2 mL of the desired inoculum in sterile saline or PBS with 0.1% Tween 20 via the lateral tail vein.
- A heat lamp, a heated box, and/or alcohol wipe may be used to dilate the vein for better visualization.
- If necessary, briefly apply slight pressure over injection site to prevent bleeding.
- Following successful inoculation, return the mice to their cages.

Monitoring of Animals Post-Inoculation:

- Following inoculation, mice should be monitored at least twice daily throughout the course of the experiment to prevent and minimize unnecessary pain and distress. Moribund animals will be identified by the following criteria:

    1. Ruffled and/or matted fur
    2. Weight loss (e.g., >20%)
    3. Hypothermia (cool to touch)
    4. Decreased activity
    5. Hunched posture
    6. Inability to eat or drink
    7. Torticollis or barrel rolling

  Any animal displaying more than one of these criteria should be humanely euthanized using at least two forms of approved euthanasia (e.g., 5% isoflurane

or pentobarbital anesthesia followed by exsanguination via cardiac puncture and cervical dislocation).

Antifungal Therapy:

- To evaluate the effects of therapy, initiate antifungal treatment after intravenous inoculation. In order to allow for the establishment of disease, begin therapy the day after inoculation (~24 hours later).
- Treatment groups typically consist of the following:
    1. Placebo controls (either saline or an excipient used to dissolve or suspend one of the positive comparators)
    2. Test compound
    3. Positive control (e.g., fluconazole, caspofungin)
- Examples of doses and dosing calculations for fluconazole and caspofungin are given at the end of this SOP.

Outcome Measures: Outcome measures of antifungal therapy that are commonly used include reductions in tissue fungal burden at a pre-specified time point and survival. Daily weights may be recorded and changes in weight can also be used as an outcome measure.

Fungal Burden. Fungal burden should be measured at a pre-specified time point following the initiation of antifungal therapy. To help control for antifungal carry-over, this time point should be at least one day after antifungal therapy is stopped (e.g., day 6 if therapy is on days 1 - 5, or day 8 if therapy is on days 1 - 7).

- At the pre-specified time point, aseptically collect the desired target organ(s) (e.g., kidneys, liver, spleen, and/or brains), and record the weight of each organ for each animal.
- Place the organs into an appropriate volume of sterile saline or PBS (e.g., 2 mL) and homogenize using either a tissue grinder or tissue homogenizer.
- Prepare appropriate dilutions (e.g., 1:10, 1:100, 1:1000) in sterile saline or PBS and plate an appropriate volume (e.g., 0.1 - 0.2 mL) of each onto Sabouraud dextrose agar plate. This may be done in duplicate.
    - Antibiotics may be included to prevent bacterial contamination. These may be added to either the sterile saline or PBS used to prepare the homogenates (e.g., chloramphenicol at 50 µg/mL and gentamicin at 0.8 µg/mL) or added to the plates onto which the homogenates are plated (e.g., chloramphenicol at 50 µg/mL).
- Incubate the plates at 37°C for at least 48 hours and count the number of colonies for each dilution. Calculate the number of colony-forming units (CFU)/gram of tissue.
    - A longer period of incubation may be used (72 hours) to allow for the growth of cells damaged but not killed by antifungals.

Survival. After the discontinuation of antifungal therapy, monitor the mice at least twice daily or as regularly as the clinical condition dictates until the desired study endpoint is reached (e.g., day 21 post-inoculation).

- Any mouse that appears moribund using the criteria specified above should be humanely euthanized. Record the death as occurring the next day.

Examples of Doses and Dosing Calculations for Caspofungin and Fluconazole

Caspofungin (Cancidas, Merck NDC# 0006-3822-10)
Reconstitute vial of caspofungin acetate powder for injection

- Allow refrigerated 50 mg vial to equilibrate to room temperature
- Aseptically add 10.8 mL of sterile water for injection to the vial
- Gently mix vial until a clear solution is obtained (do not vortex)
- Reconstituted vial 50 mg vial = 5 mg/mL

Multiply average weight of mice by the dose to determine the amount of drug to administer to each animal (e.g. 1 mg/kg x 0.025 kg = 0.025 mg)

Divide the amount of drug to be administered to each mouse by the volume that will be administered (e.g., 0.025 mg/0.2 mL = 0.125 mg/mL)

Calculate the total volume needed to dose all of the mice (e.g., 4 mL for 20 mice; plus 1 mL overage = 5 mL)

To calculate the volume to remove from the reconstituted vial and the volume needed for the dilution use the formula C1V1 = C2V2

- C1 = concentration of reconstituted vial
- V1 = volume to remove from reconstituted vial
- C2 = concentration of solution to be administered to mice
- V2 = total volume needed to dose all mice

For example:

(5 mg/mL)(V1) = (0.125 mg/mL)(5 mL)
V1 = [(0.125 mg/mL)(5 mL)]/5 mg/mL = 0.125 mL

Remove 0.125 mL from reconstituted vial and add to 4.875 mL sterile water for injection (total volume = 0.125 mL + 4.875 mL = 5 mL)

Gently mix and administer by intraperitoneal injection

*We will refrigerate the reconstituted vial for up to 1 week and use the same vial for an entire week of dosing removing daily the needed volume for the days dosing.

Fluconazole (Diflucan, Pfizer)

- Use fluconazole for injection (100 mg/50 mL vial = 2 mg/mL concentration)
- Various manufacturers make this product (e.g. Sagnet Pharmaceutical; NDC# 25021-113-02)
- Store either refrigerated or at room temperature (do not freeze)

Multiply average weight of mice by the dose to determine the amount of drug to administer to each animal (e.g. 10 mg/kg x 0.025 kg = 0.25 mg)

Divide the amount of drug to be administered to each mouse by the volume that will be administered (e.g., 0.25 mg/0.2 mL = 1.25 mg/mL)

Calculate the total volume needed to dose all of the mice (e.g., 20 mL for 20 mice; plus 10 mL overage = 30 mL)

- For fluconazole, we prepare enough for the entire dosing period (e.g., 5 to 7 days of once daily dosing)

To calculate the volume to remove from the reconstituted vial and the volume needed for the dilution use the formula C1V1 = C2V2

- C1 = concentration of reconstituted vial
- V1 = volume to remove from reconstituted vial
- C2 = concentration of solution to be administered to mice
- V2 = total volume needed to dose all mice

For example:

(2 mg/mL)(V1) = (1.25 mg/mL)(30 mL)
V1 = [(1.25 mg/mL)(30 mL)]/2 mg/mL = 18.75 mL

Remove 18.75 mL from reconstituted vial and add to 31.25 mL of sterile water for injection (total volume = 31.25 mL + 18.75 mL = 50 mL)

Gently mix and administer by oral gavage

*As stated above, for fluconazole we will prepare enough to dose all of the mice for the entire treatment period and store the fluconazole in the refrigerator.

**10 mg/kg is the largest dose of fluconazole that can be given by oral gavage using a volume of 0.2 mL. This is due to the limited concentration of the IV formulation of fluconazole (2 mg/mL). If higher doses need to be administered (e.g., 20 mg/kg), then the volume of the oral gavage must be increased (e.g., 0.3 - 0.4 mL).

References:

1. CLSI. Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved standard - Third Edition. Wayne, PA: Clinical and Laboratory Standards Institute, **2008** CLSI document M27-A3.
2. Graybill JR, Najvar LK, Holmberg JD, Luther MF. Fluconazole, D0870, and flucytosine treatment of disseminated Candida tropicalis infections in mice. Antimicrob Agents Chemother **1995**; 39:924-9.