Standard Operating Procedure (SOP)
***Scedosporium apiospermum* Murine Invasive Scedosporiosis Model**
NIH/NIAID Task Order A01

Isolate:

- *Scedosporium apiospermum* clinical isolate UTHSCSA DI16-478, originally cultured from bronchial alveolar lavage fluid. The species identity was confirmed by morphologic/phenotypic characteristics and DNA sequence analysis of the ITS domain of rRNA and the calmodulin gene.
- MICs at 72 hrs performed by the CLSI M38-A2 method and results are reported in µg/ml [1].
    - Amphotericin B 1
    - Posaconazole 1
    - Voriconazole 0.25
    - Micafungin 0.5

Mice:
Outbred male ICR (CD-1) mice have been evaluated and used in this model of invasive scedosporiosis. [2-4]

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 pharmaceutical grade cyclophosphamide at 200 mg/kg is used to render the mice neutropenic.

- Cyclophosphamide (Cytoxan 500 mg/vial, NDC# 0781-3233-94, Sandoz) should be dissolved by the addition of 25 ml of sterile water to a concentration of 20 mg/ml to the vial.
- Adjust to the necessary volume of 0.2 ml-0.3 ml/mouse intraperitoneally one day prior to inoculation to administer the appropriate dose
- To prevent bacterial super-infection and deaths in the immunosuppressed mice, mice received antibacterial prophylaxis consisting of enrofloxacin at 50 ppm in the mice's drinking water.

Inoculum Preparation and Quantification:

- Subculture the *S. apiospermum* strain at 37°C on 10 potato dextrose agar plates prior to *in vivo* use ten days before the planned day of infection.
- On the day of inoculation, add 5 ml of sterile saline with 0.1% Tween 20 to the plate and scrape the surface of the plate with a 10 µl inocula loop. Collect saline with the cells into high speed centrifuge tubes, which is 2-3 tubes. Spin at high

speed centrifugation (15,000 rpm) for 15 minutes, at 4°C with no brake in a Sorval-ss-34 rotor.

- After the spin, decant the supernatant from all tubes. Resuspend one tube in 2 mL sterile saline with 0.1% Tween 20, collect 2 mL and add to second centrifuge tube containing only the pellet and resuspend. Repeat as necessary until all pellets are resuspended into one tube.
- Prepare dilutions of the cells in sterile saline or phosphate buffered saline (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. 6 x10^4 cells/mL for survival studies or 3 x 10^4 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 potato dextrose agar. Incubate the plates at 37°C and count the number of colonies the 72 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 (~16 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., posaconazole or liposomal amphotericin B)
- Examples of doses and dosing calculations for posaconazole and liposomal amphotericin B 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 (day 8 since 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 potato 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 72 hours and count the number of colonies for each dilution. Calculate the number of colony-forming units (CFU)/gram of tissue [5].
    - A longer period of incubation may be used (96 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 Posaconazole

Posaconazole (Noxafil, Merck & Co.) NDC# 0085-1328-01

- Use posaconazole oral suspension (200 mg/5 mL vial = 40 mg/mL concentration)
- Store 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. 20 mg/kg x 0.025 kg = 0.50 mg)

Divide the amount of drug to be administered to each mouse by the volume that will be administered (e.g., 0.5 mg/0.2 mL = 2.50 mg/mL)

Calculate the total volume needed to dose all of the mice (e.g., 30 mL for 10 mice; plus 10 mL overage = 40 mL)

- We prepare enough for the entire dosing period (e.g., 7 days of twice 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:

(40 mg/mL)(V1) = (2.5 mg/mL)(40 mL)
V1 = [(2.50 mg/mL)(40 mL)]/40 mg/mL = 2.5 mL

Remove 2.5 mL from posaconazole bottle and add to 37.5 mL of sterile water for injection (total volume = 2.5 mL + 37.75 mL = 40 mL)

Gently mix and administer by oral gavage

*As stated above, for posaconazole we will prepare enough to dose all of the mice for the entire treatment period and store the posaconazole at room temperature.

Examples of Doses and Dosing Calculations for liposomal amphotericin B

Liposomal amphotericin B (AmBisome, Gilead Sciences.) NDC# 0469-3051-30

Use liposomal amphotericin B for injection (50 mg)
Reconstitute vial of liposomal amphotericin B (LAMB) powder for injection

- Aseptically add 12 mL of sterile water for injection to the vial
- Immediately after the addition of water, shake the vial vigorously for 30 seconds to completely disperse the LAMB.
- Filter the reconstituted LAMB using a syringe and the filter provided into a 15 mL conical tube wrapped in foil or 15 mL black tube to protect from light.
- Reconstituted vial = 4 mg/mL
- Store at temperatures between 2-8°C. Do not freeze.
- Dilutions will be prepared using 5% dextrose.
- Prepare daily prior to administering dose.

Multiply average weight of mice by the dose to determine the amount of drug to administer to each animal (e.g. 20 mg/kg x 0.025 kg = 0.50 mg)

Divide the amount of drug to be administered to each mouse by the volume that will be administered (e.g., 0.5 mg/0.2 mL = 2.50 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:

(4 mg/mL)(V1) = (2.5 mg/mL)(5 mL)
V1 = [(2.50 mg/mL)(5 mL)]/4 mg/mL = 3.125 mL

Remove 3.125 mL from liposomal amphotericin B and add to 1.875 mL of 5% dextrose for injection (total volume = 3.125 mL + 1.875 mL = 5 mL)

Gently mix and administer by intravenous injection. Discard any remaining diluted LAMB.

References:

1. CLSI. 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi: Approved standard - Second edition. Document M38-A2. Clinical and laboratory Standards Institute, Wayne, Pennsylvania
2. Gonzalez GM, Tijerina R, Najvar L, Rinaldi M, Yeh IT, GraybillJR. 2002. Experimental murine model of disseminated Pseudallescheria infection. Med Mycol **40:**243-248.
3. Gonzalez GM, Tijerina R, Najvar LK, Bocanegra, R, Rinaldi MG, Loebenberg D, Graybill JR. 2003. Activity of posaconazole against Pseudallescheria boydii: in vitro and in vivo assays. Antimicrob Agents Chemother **47:**1436-1438.
4. Bocanegra R, Najvar LK, Hernandez S, McCarthy DI, Graybill JR. 2005. Caspofungin and liposomal amphotericin B therapy of experimental murine scedosporiosis. Antimicrob Agents Chemother **49:**5139-5141.
5. Sheppard DC, Graybill JR, Najvar LK, Chiang LY, Doedt T, Kirkpatrick WR, Bocanegra R, Vallor AC, Patterson TF, Filler SG. 2006. Standardization of an experimental murine model of invasive pulmonary aspergillosis. Antimicrob Agents Chemother. **50:**3501-3503.

Appendix

Evaluation of Earlier Treatment Initiation on the *In vivo* Efficacy of Posaconazole in a Murine Model of Scedosporiosis

OVERVIEW & OBJECTIVE:

Scedosporiosis is an opportunistic fungal infection that is caused by different *Scedosporium* species, including *S. apiospermum, S. boydii, S. aurantiacum, S. dehoogii, S. minutispora, S. desertorum* and the recently reclassified *Lomentospora (S.) prolificans* (1, 2). These are ubiquitous moulds that are found in soil, polluted water, sewage, and manure (3, 4). Some of these species have now been reclassified into the genus *Scedosporium* replacing that of *Pseudallescheria* (e.g., *P. boydii, P. minutispora,* and *P. desertorum*) due to the results of phylogenetic analysis (5). In immunocompetent patients, these fungi are frequent causes of mycetomas, which are chronic, tumor-like infections of subcutaneous tissue and contiguous bone with draining sinuses, secondary to traumatic inoculation (3, 4). While infections are often restricted to bone and soft tissues in otherwise healthy individuals, *Scedosporium* species are capable of deeply invasive and disseminated infections in immunocompromised host (6), and these fungi are increasingly recognized as causes of breakthrough infections on antifungal therapy, especially amphotericin B, in persistently neutropenic and/ or lymphopenic patients and transplant recipients (7-14). In addition, dissemination to the central nervous system can occur in immunocompetent survivors of near-drowning events (2, 3). Unfortunately, most *Scedosporium* infections in immunocompromised patients are disseminated by the time diagnosis is established (7), which portends a poor prognosis especially in patients whose immune systems fail to recover (7, 8).

Although recent progress has been made in the treatment of infections caused by *Candida* and *Aspergillus* species with the introduction of the echinocandins and the triazoles voriconazole, posaconazole, and isavuconazole, these antifungals have modest to minimal activity at clinically relevant concentrations against *Scedosporium* species (4, 15). Similarly, amphotericin B has limited activity against *Scedosporium* species (16, 17), and its clinical utility is further limited by adverse effects and toxicities, including significant nephrotoxicity. In addition, *Lomentospora prolificans*, which was recently moved from the genus *Scedosporium* due to clear phylogenetic and morphologic differences from *Scedosporium* species, demonstrates intrinsic resistance to all clinically available antifungal agents (5). Thus, there is a clear need for the development of new therapeutic candidates and novel treatment strategies to combat infections caused by *Scedosporium* species and *L. prolificans.*

The **objective** of this study was to evaluate the effect of earlier initiation of treatment on the *in vivo* efficacy of posaconazole in a disseminated model of scedosporiosis.

APPROACH:

We utilized our neutropenic murine model of disseminated invasive scedosporiosis with which we have experience. Mice were inoculated via the tail vein due to the reproducibility of this route of inoculation, and the clinical significance of disseminated infection in immunosuppressed hosts. Disseminated models of invasive fungal infections have also proven very useful in evaluating the *in vivo* efficacy of therapeutic candidates and novel treatment strategies during pre-clinical development.

Mouse Strain - Outbred male ICR mice weighing ~28 grams were used. Mice were housed 5 per cage and had access to food and water *ad libitum*. Throughout the course of the experiments, animals were monitored at least twice daily to prevent and minimize unnecessary pain and distress. Any animal that appeared moribund prior to the scheduled endpoint was euthanized. Moribund animals were identified by the following criteria:

1. Ruffled/matted fur
2. Hunched posture
3. Weight loss (e.g., >20%)
4. Hypothermia (cool to touch)
5. Hyper-/hypoventilation
6. Inability to eat or drink

Any animal demonstrating > 1 of these criteria was euthanized by isoflurane anesthesia followed by cervical dislocation.

Immune Status – Mice were rendered neutropenic by intraperitoneal administration of a single dose of pharmaceutical grade cyclophosphamide 200 mg/kg administered one day prior to inoculation (day -1). To prevent bacterial super-infection and deaths in the immunosuppressed mice, mice received antibacterial prophylaxis consisting of enrofloxacin at 50 ppm in the mice's drinking water.

Isolate and Intravenous Inoculation – We used a contemporary strain of *S. apiospermum* due to the higher prevalence of this species in immunocompromised hosts, as well as poor response rates reported against invasive disease. The isolate we used, UTHSCSA DI16-478, was cultured from bronchial alveolar lavage fluid and received by the Fungus Testing Laboratory at UT Health San Antonio for clinical testing. The species identity was confirmed by morphologic/phenotypic characteristics and DNA sequence analysis of the ITS domain of rRNA and the calmodulin gene. The isolate has been maintained in a frozen stock in the Fungus Testing Laboratory.

Antifungal Treatment - To evaluate the *in vivo* efficacy of posaconazole, therapy began at different time points post-inoculation (i.e., 4, 8, 16, and 24 hours) and continued through day 7. In total, three survival arms and one fungal burden arm were included. Treatment groups consisted of a placebo control and posaconazole (20 mg/kg BID) administered by oral gavage, respectively. Treatment groups and treatment initiation times are shown below and the number of animals in each group are shown in the **Table 1**.

Outcome Measures – Two primary outcome measures were used to demonstrate the validity of the immunosuppressed invasive scedosporiosis model. These included:

1. Survival – In survival experiments, mice were monitored at least two times daily until day 21,14 days after therapy had been discontinued in the treatment studies to allow for adequate washout of the antifungal agents. To prevent and minimize unnecessary pain or distress, any animal that appeared moribund (by the criteria outlined in above) prior to the schedule endpoint was humanely euthanized as described above.
2. Tissue Burden – Tissue fungal burden was measured using enumeration of colony-forming units in kidneys (primary target organ) and in the lungs and brains. Fungal burden was measured in the survival arm at the pre-specified endpoint for survival (day 21 post-inoculation), and as mice succumbed to infection, and in the fungal burden arm on day 8 post-inoculation. Colony-forming units were also measured at various time points post-inoculation (i.e., 4, 8, and 24 hours) to establish fungal burden prior to the initiation of antifungal therapy. Tissues from each animal were weighed, placed into sterile saline containing gentamicin and chloramphenicol, homogenized, and the number of CFU/gram of tissue determined.

Table 1. Treatment groups and number of mice per group in survival arms.

Group	Survival Experiment #1	Survival Experiment #2	Survival Experiment #3	Fungal Burden Experiment #1
4 hour fungal burden determination	5	5	5	5
8 hour fungal burden determination	5	5	5	5
24 hour fungal burden determination	5	5	5	5
Placebo (Vehicle) Controls PO BID	10	10	10	10
Posaconazole 20 mg/kg PO BID at 4 hrs	10	10	10	10
Posaconazole 20 mg/kg PO BID at 8 hrs	10	10	10	10
Posaconazole 20 mg/kg PO BID at 16 hrs	10	10	10	10
Posaconazole 20 mg/kg PO BID at 24 hrs	10	10	10	10
Uninfected Controls	5	5	5	5
Total Animals	**70**	**70**	**70**	**70**

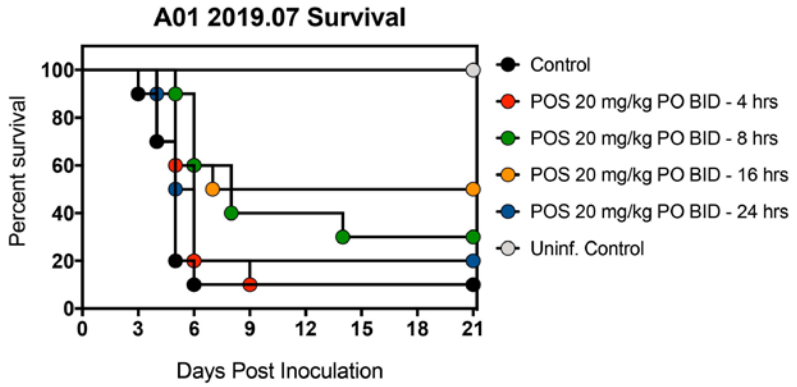
Data Analysis – Survival was plotted by Kaplan-Meier analysis and differences in the median survival time and percent survival were analyzed by the log-rank test and chi-square test, respectively. Differences in kidney, lung, and brain fungal burden were compared by ANOVA with Tukey's post-test for multiple comparisons. A p-value < 0.05 was considered statistically significant for all comparisons.

RESULTS:

Survival - In the initial survival study (A01 2019.07), earlier initiation of posaconazole therapy (8 and 16 hours post-inoculation) was associated with longer median survival compared to vehicle control. In contrast, no differences in median survival compared to control was observed when therapy was initiated either at 24 hours or 4 hours (Figure 1). The starting inoculum achieved in this study was 6.8 x 10^4 CFU/mouse.

A second survival study (A01 2019.08) was conducted. However, the starting inoculum that was achieved was markedly lower than in the first study (4.6 x 10^3 CFU/mouse). Because of this, median survival in the vehicle control group and all treatment groups was longer than observed in the first study, and no significant differences between treatment groups and vehicle control were found (Figure 2).

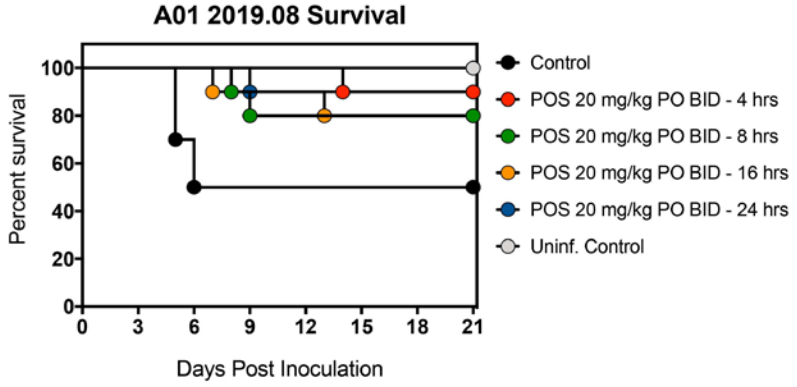
Figure 1. Study A01 2019.07 survival curves in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Median Survival	5 days	6 days	8 days p = 0.0144	14 days p = 0.0051	5.5 days	>21 days
Percent Survival	10	10	30	50	20	100

p-value vs. Control

Figure 2. Study A01 2019.08 survival curves in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



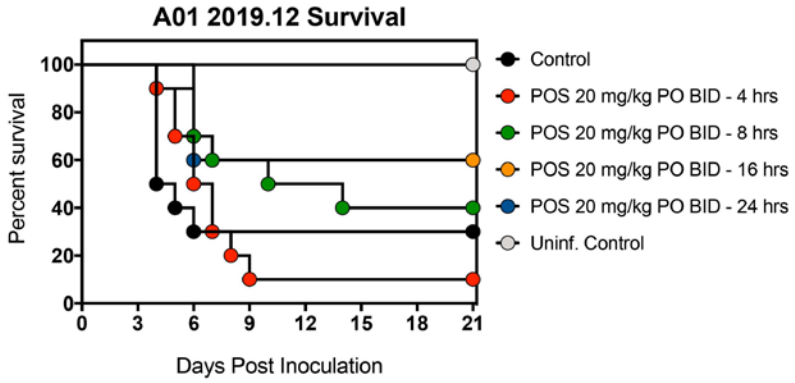
Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Median Survival	13.5 days	>21 days	>21 days	>21 days	>21 days	>21 days
Percent Survival	50	90	80	80	90	100

p-value vs. Control

A third survival arm study was conducted, and these results are shown in Figure 3. As seen in the first survival experiment, earlier initiation of posaconazole therapy (16 hours post-inoculation) was associated with longer median survival compared to vehicle control. In contrast, no differences in median survival compared to control was observed when therapy was initiated either at 4 hours or 8 hours. Interestingly, when posaconazole was started at 24 hours post-inoculation in this study, survival was significantly improved

compared to control, and these results is different than what was observed in the first study. The starting inoculum achieved in this study was 6.4 x 10^4 CFU/mouse, which is similar to that of the first study (6.8 x 10^4 CFU/mouse). Figure 4 shows a side-by-side comparison of studies 2019.07 and 2019.12.

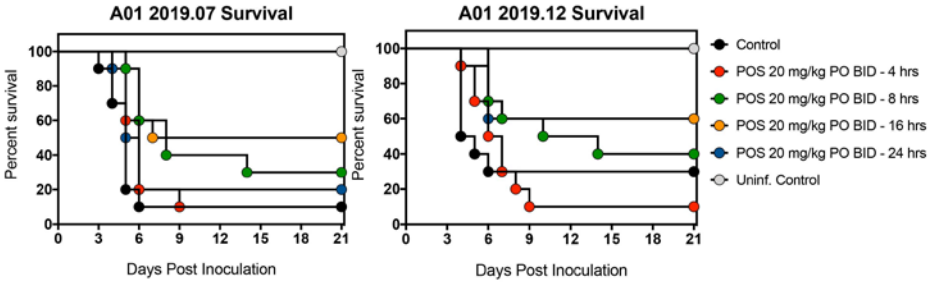
Figure 3. Study A01 2019.12 survival curves in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Median Survival	4.5 days	6.5 days	12 days	>21 days p = 0.0569	>21 days p = 0.0860	>21 days
Percent Survival	30%	10%	40%	60%	60%	100%

p-value vs. Control

Figure 4. Comparison of survival curves for studies A01 2019.07 and A01 2019.12 in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.

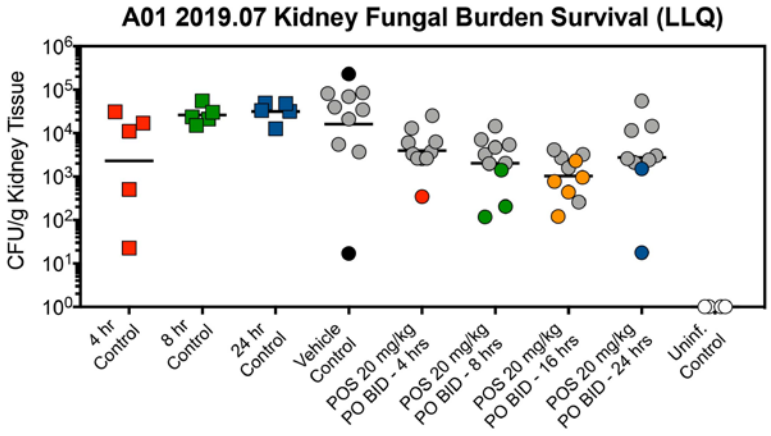


Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Median Survival 2019.07	5 days	6 days	8 days p = 0.0144	14 days p = 0.0051	5.5 days	>21 days
Median Survival 2019.12	4.5 days	6.5 days	12 days	>21 days p = 0.0569	>21 days p = 0.0860	>21 days
Percent Survival 2019.07	10%	10%	30%	50%	20%	100%
Percent Survival 2019.12	30%	10%	40%	60%	60%	100%

Fungal Burden Survival Arm - Fungal burden was also assessed in the kidneys, lungs, and brains of each survival study. Figure 5 shows the survival results of the first study. Similar to the survival results, significant reductions in kidney fungal burden were observed in mice in which posaconazole was initiated at 8 or 16 hours post-inoculation, while the differences in CFU counts were observed between the vehicle control group and posaconazole groups when treatment was started at 4 or 24 hours after inoculation (Figure 5).

Kidney fungal burden was also measured in the second study. However, due to the lower starting inoculum achieved, no differences were observed among any of the posaconazole groups compared to vehicle control (Figure 6).

Figure 5. Study A01 2019.07 kidney fungal burden (CFU/g) in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.

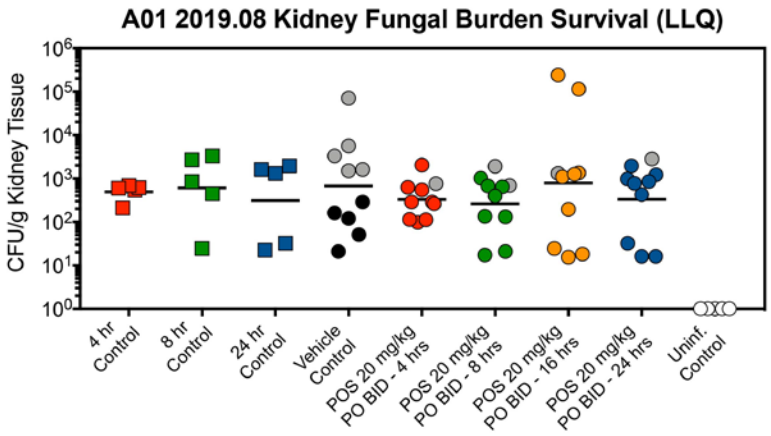


Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD)	4.20 (1.18)	3.60 (0.49)	3.31 (0.66) p = 0.0547	3.01 (0.51) p = 0.0067	3.44 (0.97)	0 (0)

p-value vs. Control

Moribund/Succumbed to Infection

Figure 6. Study A01 2019.08 kidney fungal burden (CFU/g) in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



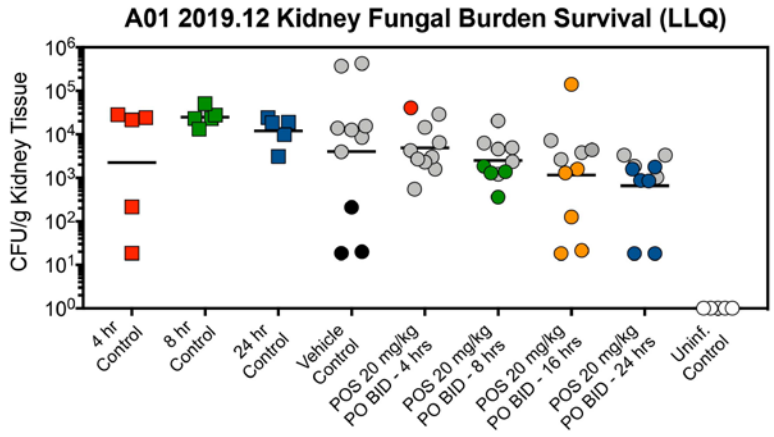
Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD)	2.83 (1.07)	2.52 (0.42)	2.42 (0.70)	2.90 (1.46)	2.52 (0.87)	0 (0)

p-value vs. Control

Moribund/Succumbed to Infection

Kidney fungal burden results from the third survival study are shown in Figure 7, and comparisons between the first and third survival studies are shown in Figure 8. Similar to the first survival study, modest reductions in fungal burden were observed when posaconazole was initiated at 8 and 16 hours post-inoculation, but not at 4 hours. In contrast to the first survival study, reductions were also observed when posaconazole was started 24 hours post-inoculation. None of the differences in CFU counts between the posaconazole groups and vehicle control were statistically significant, which may be due to a lower mean value for the vehicle control group in the third study.

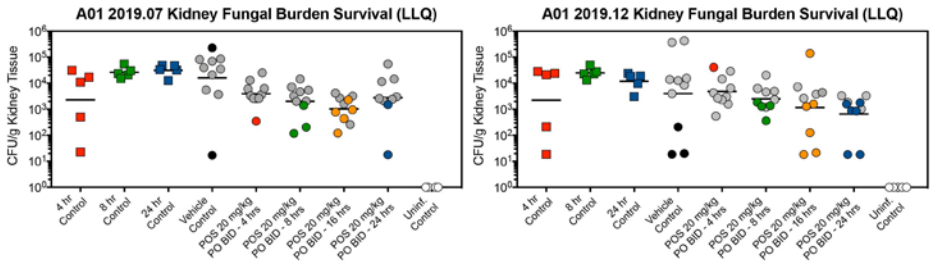
Figure 7. Study A01 2019.12 kidney fungal burden (CFU/g) in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD)	3.60 (1.54)	3.69 (0.58)	3.40 (0.48)	3.06 (1.20)	2.82 (0.85)	0 (0)

Moribund/Succumbed to Infection

Figure 8. Comparison of kidney fungal burden for survival studies A01 2019.07 and A01 2019.12 in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



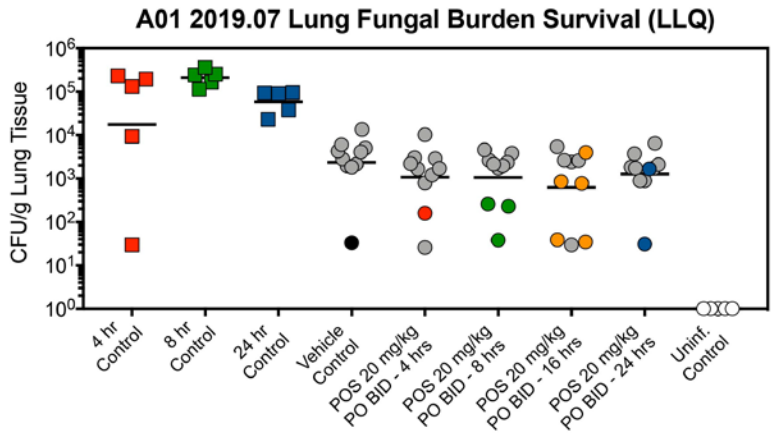
Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD) 2019.07	4.20 (1.18)	3.60 (0.49)	3.31 (0.66) p = 0.0547	3.01 (0.51) p = 0.0067	3.44 (0.97)	0 (0)
Mean $\log_{10}$ CFU/g (SD) 2019.12	3.60 (1.54)	3.69 (0.58)	3.40 (0.48)	3.06 (1.20)	2.82 (0.85)	0 (0)

Fungal burden was also assessed within the lungs and brains of mice each of the survival studies. In the first study, no differences in CFU counts were observed in either organ between the vehicle control group and the posaconazole groups (Figures 9 and 10), although there appeared to be a trend towards a reduction in brain fungal burden when posaconazole was initiated 16 hours post-inoculation (Figure 10). However, marked variability was also observed in this group.

Due to the lower starting inoculum achieved in the second study, no differences in fungal burden were observed in the lungs or brains of mice treated with posaconazole at different initiation time points and the vehicle control group (Figures 11 and 12). In addition, marked variability was observed in brain CFU counts for all groups (Figure 12).

In the third survival study, similar results in fungal burden within the lung and brain tissues were observed as compared to the first survival study (Figures 13 and 14). Comparisons between the first and third survival studies are shown in Figures 15 and 16.

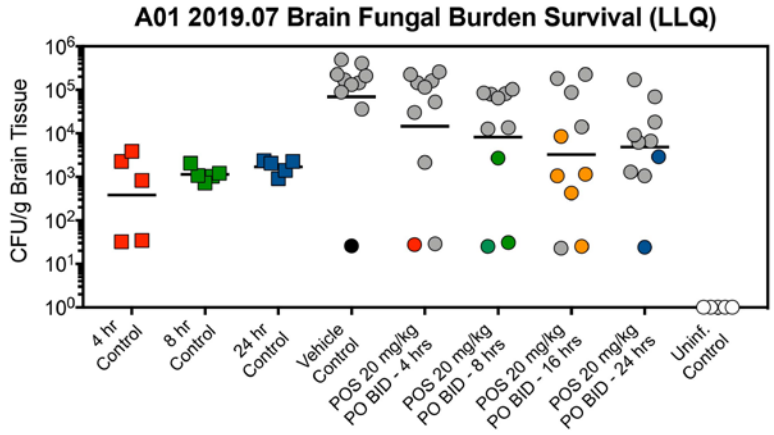
Figure 9. Study A01 2019.07 lung fungal burden (CFU/g) in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD)	3.37 (0.70)	3.03 (0.73)	3.02 (0.67)	2.80 (0.91)	3.10 (0.62)	0 (0)

Moribund/Succumbed to Infection

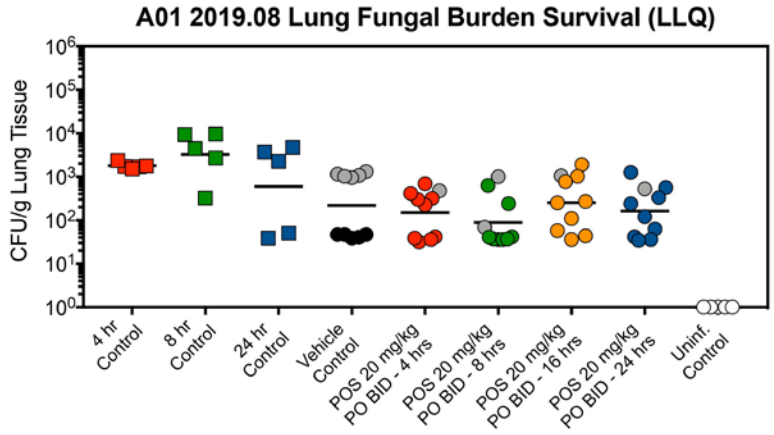
Figure 10. Study A01 2019.07 brain fungal burden (CFU/g) in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



Group	Vehicle Control	POS 20 m)g/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD)	4.84 (1.25)	4.16 (1.55)	3.91 (1.39)	3.51 (1.47)	3.69 (1.07)	0 (0)

Moribund/Succumbed to Infection

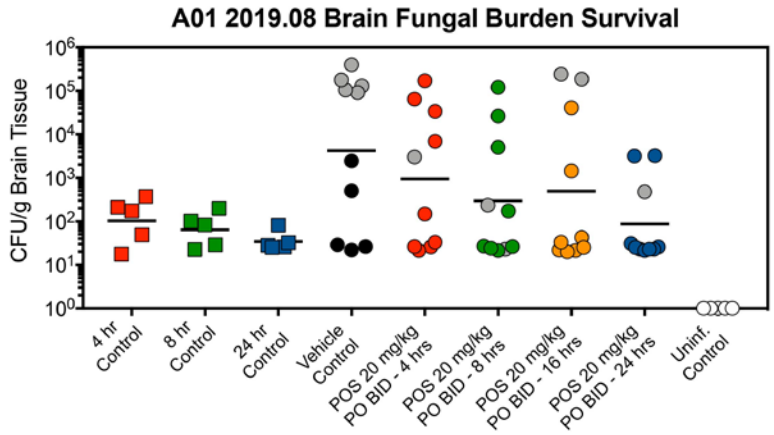
Figure 11. Study A01 2019.08 lung fungal burden (CFU/g) in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD)	2.34 (0.74)	2.17 (0.54)	1.95 (0.56)	2.40 (0.63)	2.21 (0.56)	0 (0)

Moribund/Succumbed to Infection

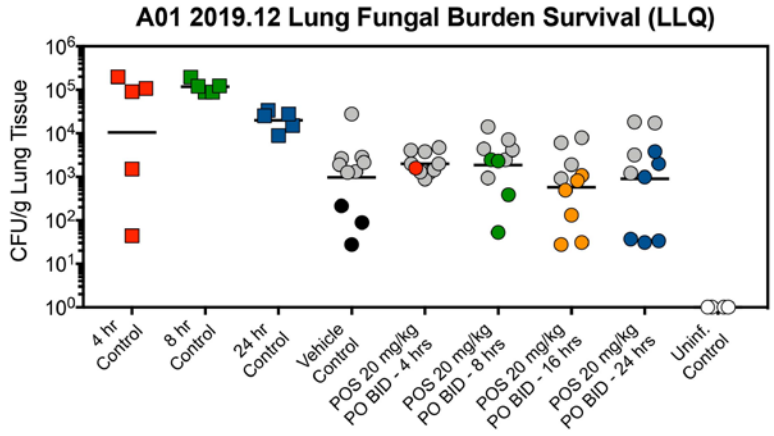
Figure 12. Study A01 2019.08 brain fungal burden (CFU/g) in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



Group	Vehicle Control	POS 20 m)g/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD)	3.62 (1.77)	2.97 (1.57)	2.47 (1.42)	2.69 (1.75)	1.94 (0.92)	0 (0)

Moribund/Succumbed to Infection

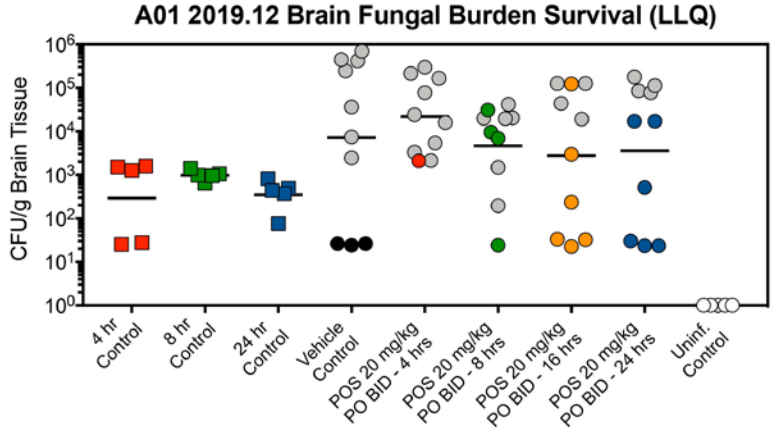
Figure 13. Study A01 2019.12 lung fungal burden (CFU/g) in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD)	2.99 (0.86)	3.30 (0.24)	3.27 (0.70)	2.76 (0.85)	2.95 (1.07)	0 (0)

Moribund/Succumbed to Infection

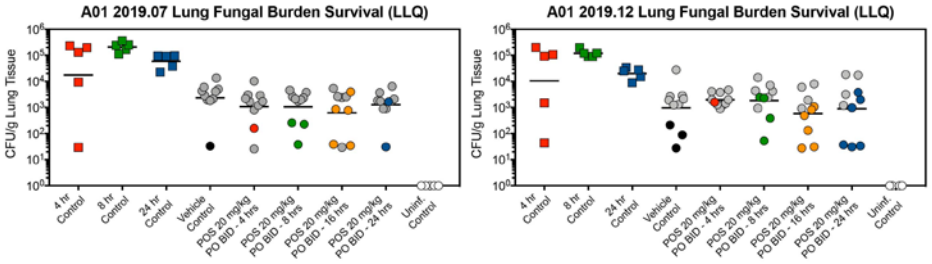
Figure 14. Study A01 2019.12 brain fungal burden (CFU/g) in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



Group	Vehicle Control	POS 20 m)g/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD)	3.85 (1.87)	4.34 (0.85)	3.67 (1.07)	3.44 (1.61)	3.55 (1.64)	0 (0)

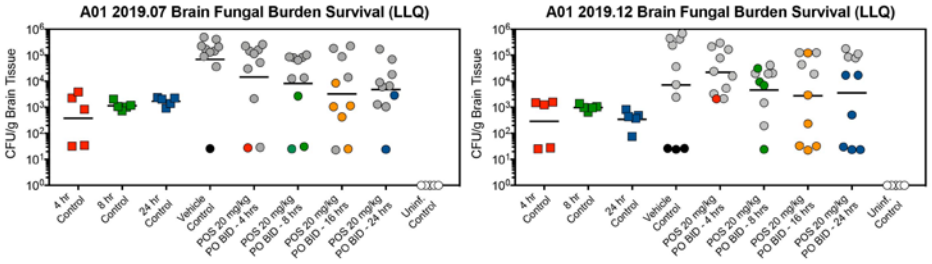
Moribund/Succumbed to Infection

Figure 15. Comparison of lung fungal burden for survival studies A01 2019.07 and A01 2019.12 in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD) 2019.07	3.37 (0.70)	3.03 (0.73)	3.02 (0.67)	2.80 (0.91)	3.10 (0.62)	0 (0)
Mean $\log_{10}$ CFU/g (SD) 2019.12	2.99 (0.86)	3.30 (0.24)	3.27 (0.70)	2.76 (0.85)	2.95 (1.07)	0 (0)

Figure 16. Comparison of brain fungal burden for survival studies A01 2019.07 and A01 2019.12 in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



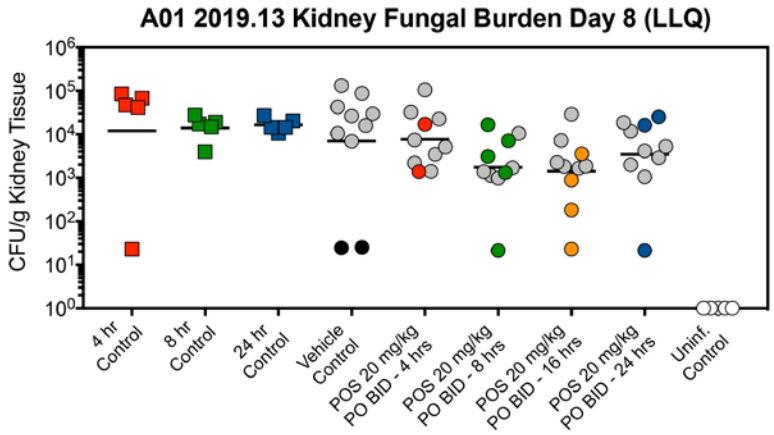
Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD) 2019.07	4.84 (1.25)	4.16 (1.55)	3.91 (1.39)	3.51 (1.47)	3.69 (1.07)	0 (0)
Mean $\log_{10}$ CFU/g (SD) 2019.12	3.85 (1.87)	4.34 (0.85)	3.67 (1.07)	3.44 (1.61)	3.55 (1.64)	0 (0)

Fungal Burden Arm Results - In the last study that was conducted, fungal burden was assessed on day 8 post-inoculation following different initiation times of posaconazole. The starting inoculum achieved in this study was 3.1 x 10^4 CFU/mouse, and CFU counts within the kidneys are shown in Figure 17. Reductions in CFU counts were observed in the groups in which posaconazole was initiated between 8, 16, and 24 hours post-inoculation compared to vehicle control. However, these differences were not statistically significant. No reduction in kidney fungal burden was observed in the posaconazole group in which treatment was started at 4 hours. These results are consistent with what was observed in the survival studies.

Within the lungs, reductions in fungal burden were observed in each of the posaconazole groups on day 8 post-inoculation, and these results were statistically significant in the 8, 16, and 24 hour groups, but not in mice in which treatment was started 4 hours post-inoculation (Figure 18).

In contrast, no reductions in fungal burden were observed within the brain tissue in any posaconazole group compared to vehicle control on day 8 post-inoculation (Figure 19).

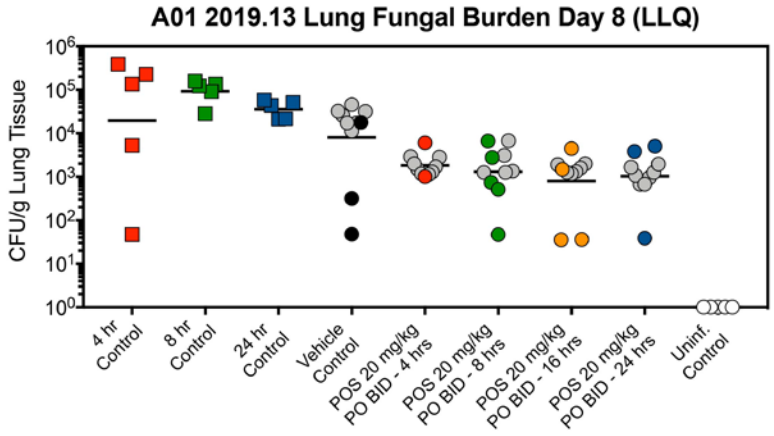
Figure 17. Study A01 2019.13 kidney fungal burden (CFU/g) on day 8 post-inoculation in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD)	3.84 (1.35)	3.89 (0.63)	3.24 (0.80)	3.15 (0.85)	3.54 (0.90)	0 (0)

● Moribund/Succumbed to Infection

Figure 18. Study A01 2019.13 lung fungal burden (CFU/g) on day 8 post-inoculation in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.

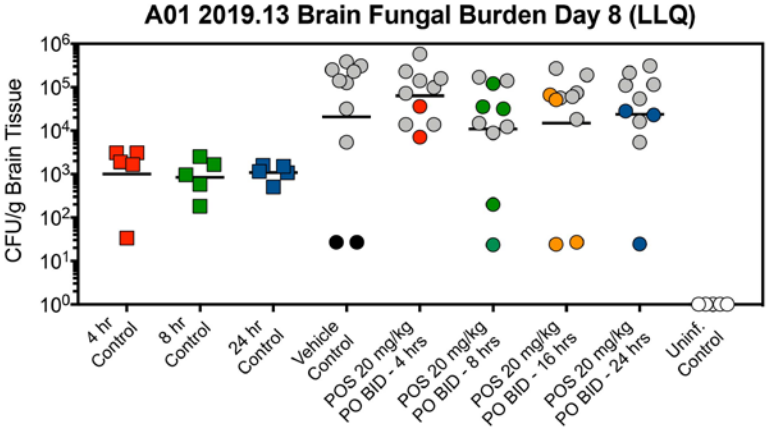


Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD)	3.90 (0.99)	3.26 (0.24)	3.12 (0.63) p = 0.0428	2.90 (0.73) p = 0.0067	3.01 (0.58) p = 0.0187	0 (0)

p-value vs. Control

● Moribund/Succumbed to Infection

Figure 19. Study A01 2019.13 brain fungal burden (CFU/g) on day 8 post-inoculation in mice infected with *S. apiospermum* and treated with posaconazole 20 mg/kg BID starting at different timepoints post-inoculation.



Group	Vehicle Control	POS 20 mg/kg 4 hrs	POS 20 mg/kg 8 hrs	POS 20 mg/kg 16 hrs	POS 20 mg/kg 24 hrs	Uninf. Control
Mean $\log_{10}$ CFU/g (SD)	4.32 (1.62)	4.80 (0.61)	4.04 (1.26))	4.17 (1.49)	4.38 (1.81)	0

● Moribund/Succumbed to Infection

SUMMARY:

Results of the first study suggested that earlier initiation of posaconazole therapy (8 or 16 hours) may be associated with improved outcomes, including improvements in median survival and reductions in kidney fungal burden, in this murine model of disseminated scedosporiosis. Variability was observed between studies in which therapy was delayed until 24 hours post-inoculation. Interestingly, initiation of therapy at 4 hours post-inoculation was consistently associated with worse outcomes compared to when treatment was started at later times.

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