APX001 and Other Gwt1 inhibitor Prodrugs are Effective in Experimental *Coccidioides immitis* Pneumonia

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ABSTRACT

Coccidioidomycosis is a systemic fungal infection caused by the inhalation of the arthroconidia of either of two closely related dimorphic fungi, *Coccidioides immitis*, and *C. posadasii* that are endemic in the southwestern US and other areas in the Western Hemisphere. Chronic cavitary pulmonary infections and extra-pulmonary sites of infection are very difficult to treat and often require life-long azole therapy. ~~to suppress the growth of spherules, the tissue form of these fungi~~. APX001A is the first in a new class of broad spectrum antifungal agents which inhibit Gwt1, an enzyme which is required for localization of glycosylphosphatidyl inositol (GPI)-anchored mannoproteins in fungi. APX001A and several analogs were highly active against clinical isolates of *Coccidioides*, inhibiting hyphal growth at low nanogram/ml concentrations. APX001 is the N-phosphonooxymethyl prodrug of APX001A, currently in clinical trials for the treatment of invasive fungal infections. Mice were treated orally once-daily with 26 mg/kg/day of APX001 and the prodrug analog APX2097, two hours after administration of the pan-cytochrome P450 inhibitor 1-aminobenzotriazole, which was used to enhance drug half-life and exposures to more closely mimic human pharmacokinetics of APX001A. Five days of treatment reduced lung colony counts by nearly 3 logs and prevented dissemination, similar to the efficacy of fluconazole dosed orally at 25 mg/kg twice daily. In a survival experiment, both APX001 and APX2097-treated mice survived significantly longer than control and fluconazole treated mice. ~~We conclude that~~ APX001 and other members of this new class of antifungal agents may offer great promise as effective therapies for coccidioidomycosis.

INTRODUCTION

Coccidioidomycosis (San Joaquin Valley Fever) is a systemic fungal infection that is endemic in the Southwestern United States from West Texas to Southern and Central California and in arid regions in Central and South America (1). The disease is caused by two closely related species, *Coccidioides immitis* and *C. posadasii* (2) both of which are dimorphic. Desert rodents [the natural host (3)] and humans become infected by inhaling arthroconidia (spores) that are aerosolized by wind. After the spores enter a mammalian host, they convert to round cells that enlarge to become spherules. The spherules are large, spherical structures that grow to a diameter of > 100 microns and reproduce by segmenting internally into hundreds of endospores that are released when the spherule ruptures. In the US, coccidioidomycosis is a reportable infection only in California and Arizona. The incidence in those two states has been increasing in recent years (4). ~~Even before the recent increase, it was estimated that there were ~150,000 new infections annually in the U.S. (5).~~ Many infections are either asymptomatic or so mild that people do not seek medical attention. However, symptomatic pneumonia can be severe and debilitating; ~5% of infections spread to extra-pulmonary sites and are extremely difficult to treat. Disseminated infection accounts for most of the deaths due to coccidioidomycosis (6). ~~The annual cost of hospitalization for this disease in California alone is in the hundreds of millions of U.S. dollars (7).~~

The first effective treatment approved in the U.S. for coccidioidomycosis was amphotericin B deoxycholate, which is quite toxic [(8)]. However, even the newer lipid formulations demonstrate toxicity. ~~Similarly,~~Although ketoconazole is FDA approved for the treatment of coccidioidomycosis~~. However, due to lesser potency compared to the newer triazoles, the potential for severe liver injuries, and inhibition of adrenal gland~~

enzymes, it is no longer recommended for treatment of coccidioidomycosis due to toxicity and lesser potency than the newer triazoles (9). Fluconazole and other triazoles are now the most frequently used drugs to treat coccidioidomycosis. Triazoles are effective treatment for most disseminated infections, but , however relapse of coccidioidomycosis is common when they are discontinued [9]. The benefits of fluconazole and itraconazole in chronic infections are not dramatic, requiring a complicated scoring system developed by the Mycoses Study Group (MSG) to show a beneficial effect (10, 11). In addition, there is recent evidence that some clinical isolates of *Coccidioides* have high MIC values for fluconazole (11). Thus, there is a need for new drugs for this infection.

In this study, we evaluated the *in vitro* and *in vivo* activity of a novel class of broad spectrum antifungal agents against *Coccidioides* spp. These compounds are structurally and mechanistically unrelated to other antifungal drugs and inhibit the highly conserved fungal enzyme Gwt1, which is required for localization of glycosylphosphatidyl inositol (GPI)-anchored mannoproteins in fungi (12-14). In *C. albicans,* these GPI-anchored mannoproteins are often components of the cell wall, are surfaced exposed, and have other diverse cellular functions (14, 15).

For assessment of *in vivo* efficacy, N-phosphonooxymethyl prodrugs of these molecules **(Fig. 1)** were synthesized in an analogous method to the synthesis of APX001 (16, 17). These prodrugs are rapidly and completely metabolized by host alkaline phosphatases to the active moieties (18-20). APX001A has a short half-life in mice (1.4 to 2.5 h) after administration of the prodrug APX001 (20), whereas Phase 1 studies in healthy volunteers have shown a half-life of 2½ days and exposures of ≥ 200 µg·h/mL (21, 22). To enable dosing regimens that more closely mimic human

pharmacokinetics, we orally administered 1-aminobenzotriazole (ABT), a nonselective suicide inhibitor of cytochrome P450 (CYP) enzymes (23), 2 h prior to the oral administration of APX molecules. Previous studies have shown that ABT extends the half-life and increases the exposure of APX001A and other related APX molecules, after administration of the corresponding prodrugs (19, 24). ABT has been shown to have no *in vitro* antifungal activity against 4 species *(Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus*, and *Scedosporium apiospermum)* when tested at concentrations up to 250 µg/ml, nor does it demonstrate synergistic effects when evaluated in combination with APX001A (Kapoor, unpublished observations).

RESULTS

In vitro* activity of Gwt1 inhibitors vs *Coccidioides. The *in vitro* activity of the active moiety APX001A was evaluated against three laboratory strains of *Coccidioides* **(Table 1).** Since there is no standardized CLSI method for *Coccidioides*, we compared the minimal effective concentration (MEC) causing abnormal hyphal growth (short abundant branching) in a microbroth dilution assay and also determined the MIC values of APX001A, fluconazole, posaconazole, and amphotericin B against *Coccidioides* arthroconidia using a microbroth serial dilution assay. The MEC value for APX001A was approximately 1-3 logs lower than the MIC value and was easier to determine precisely with no inter-observer variation **(Table 1).** The use of a MEC endpoint for APX001A and the echinocandins has been established for other molds, including *Aspergillus* species (25-27). The MIC values for posaconazole ranged between 0.03 to 0.125 µg/ml and >16 µg/ml for fluconazole, when read at the more stringent endpoint of 100% inhibition rather than the less stringent CLSI reading of 50% inhibition for azoles and other molds (28).

The activity of 33 APX001A analogs were evaluated against one strain each of *C. immitis* and *C. posadasii*. Sixteen compounds were active at levels ≤ 0.016 µg/ml (data not shown), and two of the most active compounds (APX2020, APX2041) were chosen for further analysis against a larger panel of strains that included 5 isolates each of *C. posadasii* and *C. immitis* **(Fig. 1, Table 2).** The activity of these compounds was compared to APX001A and posaconazole, one of the most potent azoles against *Coccidioides* (11). All three Gwt1 inhibitors were highly active, with geometric mean MEC values of 0.002, 0.004, and 0.008 µg/mL for APX2041, APX2020, and APX001A, respectively, while the geometric mean MIC for posaconazole was 0.125 µg/ml **(Table 2).** The ranges of MEC values for *C. immitis* appeared to be slightly lower (2 to 8-fold) than those for *C. posadasii* for the three Gwt1 inhibitors **(Table 2).**

***In vivo* activity of Gwt1 inhibitors vs *C. immitis*. (i) Activity of APX001 in a pulmonary murine coccidioidomycosis model.** A mouse model of coccidioidomycosis was used to evaluate the activity of APX001 against the pathogenic form of the fungus. B6 mice were chosen due to their genetic susceptibility to this infection (29). Thus, this model would be analogous to treating patients who are genetically predisposed to disseminated infection, the most challenging group of patients to treat. Mice were infected by inhalation of ~200 arthroconidia/mouse and treatment was initiated 7 days later in order to allow enough time for the arthroconidia to transform into spherules. Mice were then treated twice daily by oral gavage with 50 mg/kg of APX001 for 5 consecutive days. The geometric mean $\log_{10}$ CFU/g in lung and spleen in the untreated control groups were 7.91 and 3.99, respectively (**Fig. 2**). APX001 treatment reduced the lung CFU geometric mean lung CFU by nearly 2.75 logs (P =0.0011), and prevented dissemination to the spleen (P =0.0031). Brain CFU were

also examined and all 8 APX001-treated animals demonstrated complete sterilization versus <5 CFU/g brain tissue in controls (P = 0.0002) (data not shown). As further evidence for the efficacy of APX001 treatment, mice treated with APX001 did not lose weight, while the control mice lost 24% of body weight by Day 13 (P =<0.01) **(Fig. 2).**

(ii) 1-Aminobenzotriazole (ABT) has no antifungal activity in mice. Due to the short half-life of APX001A after APX001 administration in mice (1.4 to 2.5 h), and the importance of area under the curve (AUC)/MIC as the driver of efficacy (20) we concluded that BID dosing was not an optimal treatment regimen for coccidioidomycosis. To more closely mimic the long half-life (2 to 2 ½ days) observed in phase 1 clinical studies (21, 22), we evaluated the use of the pan-CYP450 inhibitor ABT in the coccidioidomycosis model. ABT had been previously shown to extend the half-life and increase the AUC of the four Gwt1 inhibitors shown in **Fig. 1** by 8.6 to 15-fold after dosing of the prodrug (19, 24).

To determine whether ABT had an antifungal or toxic effect in this model, mice were infected with ~200 arthroconidia/mouse and single daily doses of 50 mg/kg ABT were administered starting 4 days after infection and continuing for 5 days. The data in **Fig. 3** show that $\log_{10}$ CFU/lung and spleen were not significantly different from the untreated control group (P >0.2 for both), demonstrating no antifungal effect of ABT. In addition, the administration of ABT to infected mice did not significantly decrease body weight vs the vehicle control (P =0.95) (**Fig. 3**), nor cause an increase in serum alanine transaminase (ALT) or serum bilirubin (data not shown).

(iii) Efficacy of three Gwt1 inhibitor prodrugs in the treatment of pulmonary coccidioidomycosis: evaluation of CFU. The activities of three APX001 analogs were evaluated in the coccidioidomycosis mouse model. These compounds included

the N-phosphonooxymethyl prodrugs of APX2020 and APX2041, along with a third molecule APX2039 **(Fig. 1).** Although APX2039 was 2 to 4-fold less active against the *C. immitis* RS strain used in the mouse model (MEC = 0.008 µg/ml), the prodrug APX2096 had previously been shown to have improved pharmacokinetics and better efficacy in a cryptococcal meningitis model of infection (19). Mice were infected as in **Fig. 2** with ~200 arthroconidia/mouse, and treatment was initiated on Day 7 after infection as before, but in this experiment the mice were pre-treated with 50 mg/kg ABT by oral gavage 2 h prior to administration of 26 mg/kg APX prodrugs by oral gavage. This treatment regimen was continued for 5 days. Mice were weighed at the start and conclusion of the experiment and were sacrificed one day after the last dose. The reduction in fungal colony counts (CFUs) in lung and spleen upon treatment with the three respective prodrugs APX2097, APX2104, and APX2096 **(Fig. 1)** is shown in **Fig. 4.** Efficacy was observed for all three treatments as compared to the control plus ABT, as measured by significant decreases in $\log_{10}$ CFU organ (lung, P <0.0001 and spleen, P <0.01). Only the control mice lost weight and at the end of treatment they weighed significantly less than the treated mice. However, APX2096 did not reduce dissemination to the spleen as effectively as the other two derivatives and was thus not pursued further (**Fig. 4**).

We next compared the *in vivo* activities of once daily APX001 and APX2097 to the activity of fluconazole. Fluconazole (25 mg/kg), which is considered first-line therapy in the treatment of coccidioidomycosis in humans (9),was administered orally BID by gavage without ABT pretreatment. Mice were sacrificed one day after they had received treatment for 5 days for assessment of CFU/g tissue. All three treatment groups had

significantly lower CFU/lung than the control group, and all prevented dissemination to the spleen (with the exception of one mouse in each group) (**Fig. 5**). We repeated this experiment (excluding the fluconazole group) to evaluate the appearance of the spherules in the infected lungs. **Fig. 6** shows representative lung fields mice treated with ABT/glucose, APX001, and APX2097. The lung from the control mouse shows spherules in all stages of maturation and numerous free endospores, while the spherules in the APX001 and APX2097 treated mice were all small and immature, and many had been ingested by macrophages.

(iv) Efficacy of three Gwt1 inhibitor prodrugs in the treatment of pulmonary coccidioidomycosis: evaluation of survival. The same infection and dosing conditions were utilized as shown in **Fig. 5,** however the endpoint was survival 30 days after infection (18 days after the last treatment dose). As shown in **Fig. 7**, the fluconazole treated mice survived significantly longer than the control mice (P = <0.01). However, mice treated with APX001 survived significantly longer than the fluconazole treated mice (P = <0.01), and the mice treated with APX2097 survived significantly longer than the APX001-treated mice (P = <0.01). The one surviving mouse in the APX2097 group at the end of the experiment was infected.

DISCUSSION

In this study we demonstrated that oral administration of the prodrug APX001 and three other prodrug analogs were effective treatments for experimental murine coccidioidomycosis caused by *C. immitis*. To be sure that the drugs were acting on the tissue stages of this dimorphic fungus and not the arthroconidia used to infect the mice

(30), treatment was delayed until 7 days after infection. Thus the infection more closely mimicked treatment of coccidioidomycosis pneumonia, as would be seen in clinical practice. The appearance of the organisms in the APX prodrug treated mice at the end of therapy, as determined by histological analyses of lung tissue sections, was that of immature spherules, suggesting that was their stage of development when treatment began and the APX drugs prevented further maturation.

We assessed two all oral treatment regimens that led to similar reductions in fungal burden. Mice were treated either with 50 mg/kg BID of APX001, or they were pretreated with 50 mg/kg of the pan-CYP450 inhibitor ABT 2 h prior to administering the APX prodrugs at 26 mg/kg once daily. ABT prolonged the half-life and increased the exposure of the APX active moieties by 8.6 to 15-fold, so that once daily dosing with ABT achieved similar or better therapeutic benefits as multiple higher doses of the APX molecules without ABT. This is consistent with *in vivo* efficacy being a function of drug exposure, as has been observed for APX001 and its analogs in other infection models (19, 20, 24). The oral 26 mg/kg QD treatment regimen reduced colony counts as well as twice daily oral treatment with 50 mg/kg fluconazole, given for the same duration. Although fluconazole is not the most active triazole against the mold form of *Coccidioides*, it is considered first-line therapy for coccidioidomycosis (9), and is easy to administer orally in mice because it is water soluble (31).

When we compared the ability of fluconazole and the APX drugs to prolong survival after the end of therapy we found that the two Gwt1 inhibitor prodrugs APX001 and APX2097 were superior to fluconazole (P <0.01) in that they prolonged survival for many days after treatment ended (**Fig. 7)**. Although the functions of GPI-linked proteins in *Coccidioides* are still unknown, the antifungal activity of the Gwt1-inhibitors both *in*

vitro and *in vivo* implies that they are of vital importance for both the hyphal and the spherule stages of the fungus. The more prolonged survival after treatment was stopped may be due to a longer post-antifungal effect of the Gwt-1 inhibitors (32), better immune system recognition due to a loss of mannoproteins (15), or other factors. Further work is needed to determine the factors that result in the persistent activity of the Gwt1 inhibitors after treatment ended.

The APX drugs were also tested against the mold form of the fungus *in vitro.* One of the difficulties in evaluating the activity of compounds *in vitro* against dimorphic *Coccidioides* spp is the lack of standardized CLSI methodology (33). Perhaps of more significance, *in vitro* testing is done against arthroconidia that develop into hyphae under the conditions of the assay, but hyphae are not the pathogenic form of the fungus. We used a broth microdilution methodology similar to CLSI standard method for determining MEC endpoints (33), and we found the APX drugs to be highly active against the hyphal form of both species of *Coccidioides*. The MEC endpoint has been previously shown to be a reliable and reproducible method for evaluation of the activity of APX001A (formerly E1210) (25, 26) and the echinocandins (27). A caveat about the significance of MEC *in vitro* results is that the ability to prevent hyphal growth may not be directly relevant to treating infections that are due to spherules. Although one would like to test activity against spherules, since they reproduce by circumferential growth and sequential septation within the spherule (30), monitoring the effect of antifungal drugs on this stage by ordinary microscopy or changes in turbidity *in vitro* is not feasible. Therefore, we tested the drug in an *in vivo* model and preliminary morphological evidence suggests APX001A and its analogs also inhibit the growth and maturation of spherules.

Previous susceptibility testing of *Coccidioides* has been performed by broth macrodilution according to methods described in CLSI M38-A3, with MIC values read as the lowest concentration that resulted in ≥80% inhibition of growth vs the no drug control (28). Using this methodology, a recent study evaluated 377 *Coccidioides* clinical isolates and determined that the posaconazole MIC_{90} was 0.25 µg/ml. Those data are similar to the results of this study where a smaller collection of 10 strains was evaluated using a broth microdilution assay (reading 100% inhibition endpoint) and a posaconazole MIC_{90} value of 0.125 µg/ml was observed. Likewise, the previous study showed that the MIC_{90} value for fluconazole was 16 µg/ml, with 37% of clinical isolates exhibiting fluconazole MIC values of ≥16 µg/ml and 3.8% with MIC values of ≥64 µg/ml (11). In the current study, we also observed a fluconazole MIC_{90} of >16 µg/ml (**Table 1**). Although fluconazole is the most commonly used antifungal agent for *Coccidioides* infections, the use of other agents with lower MIC values such as the newer triazoles or Gwt1 inhibitor prodrugs such as APX001 may be better alternative treatment options for coccidioidomycosis *(11)*.

APX001 is a first-in-class, broad-spectrum antifungal agent that is currently in clinical development for the treatment of life-threatening invasive fungal infections. APX001 has been shown to be effective in mouse models of *Candida albicans* infections (20, 24, 34), *Candida auris (35)*, *Cryptococcus neoformans* (19) as well as *Aspergillus* and *Fusarium* (18). In addition to increased survival, reduction of colony counts of fungi in the lungs, kidney and brain tissues of infected mice has been observed, consistent with ^{14}C-APX001 studies which demonstrated wide tissue distribution in rats and monkeys, especially in tissues associated with invasive fungal infections (36). Notably, treatment with APX001 lead to a significant reduction in brain CFU in both a rabbit model of

hematogenous *Candida albicans* meningoencephalitis (37) and a mouse disseminated *Candida auris* model (35). CFU in brain were also examined in this study, and the APX001-treated group resulted in sterilization of the brain in all animals. However, the untreated control group demonstrated low CFU counts (< 5 CFU/g tissue) and thus although statistical significance was reached (P = 0.0002), the low numbers make it difficult to assess biological significance.

In this study we demonstrate that APX001A, the active moiety of APX001, has good *in vitro* activity against the mold form of *Coccidioides,* with a MEC_{90} of 0.008 µg/mL. Two additional Gwt1 inhibitor analogs, APX2020 and APX2041, demonstrated 2 to 4-fold improved activity vs APX001A with MEC_{90} values of 0.004 and 0.002 µg/mL, respectively against a panel of *C. immitis* and *C. posadasii* strains (**Table 2**). These values compare favorably with posaconazole (MIC_{90} 0.125 µg/ml), one of the triazoles that is used clinically for the treatment of coccidioidomycosis (38).

In summary, we found that APX001A and its analogs were highly active *in vitro* against both species of *Coccidioides*, and that oral administration of the corresponding prodrugs were effective treatments for pulmonary coccidioidomycosis and prevented systemic spread in a genetically susceptible mouse strain. The demonstrated efficacy against *Coccidioides,* as well as previous studies of efficacy against other yeasts and molds, provides support that APX001 is a promising new broad-spectrum antifungal agent worthy of continued investigation.

MATERIALS AND METHODS

Isolates tested and organism handling. All isolates tested were originally clinical isolates. However, *C. immitis* RS, *C. posadasii* Silvera, and *C. posadasii* C735 have been passaged for years in different laboratories. We also collected clinical isolates

from cases diagnosed in San Diego over the 24-months prior to the *in vitro* testing (**Table 2**). Standard BSL3 safety precautions were followed for all *in vitro* work.

Arthroconidia preparation. Arthroconidia were prepared as previously described (39). *Coccidioides* colonies were grown on 2x glucose-yeast extract (GYE) agar. The plates were incubated at 30°C until the mycelia covered the surface of the agar. Arthroconidia were harvested from the plate after 4-5 weeks of incubation at 25°C by adding 25 ml of saline. The plate was gently scraped using cell scraper and the fluid transferred to a 50 ml tube that was then vigorously mixed for 10 seconds and centrifuged at 3000 rpm for 10 min at 4°C. The supernatant containing floating mycelia was discarded. The pellet containing arthroconidia was re-suspended in saline and passed through 3 layers of miracloth (Calbiochem) to filter out mycelia. The strained suspension was centrifuged again, re-suspended in saline and the arthroconidia were quantitated by counting under microscope using a hemocytometer. The viability is determined by dilution plating and counting colony forming units (CFU) on GYE agar.

Reagents: APX001 is the prodrug of APX001A. APX2097 is the prodrug of APX2020, APX2104 is the prodrug of APX2041, and APX2096 is the prodrug of APX2039 (Amplyx Pharmaceuticals, San Diego, CA) (**Fig. 1).** Posaconazole and fluconazole solutions were pharmacy grade.

***In vitro* susceptibility testing.** Drug susceptibility tests were performed using a broth microdilution method according to the Clinical and Laboratory Standard Institute (CSLI) M38-A2 (28). The assay was conducted in RPMI 1640 media (Sigma) containing 0.165M morpholinepropanesulfonic acid (MOPS, Sigma) at pH 7.0. Two-fold serial dilutions of the drug were made in RPMI from the highest concentration of 16 µg/ml to the lowest of 0.016 ng/ml. Arthroconidia were diluted in RPMI media. One µl of the spore suspension was added to 99 µl of drug in one well of a 96 well U-bottom sterile

plate (Corning) to a final concentration of $5x10^4$/ml. A control well was set up with DMSO only. Each dilution of the drugs was tested in duplicate and the plates were incubated at 37°C for 2-3 days.

The plates were visually scored using a magnifying mirror to determine the MIC (100% inhibition). The MEC scores were determine by examining each well for growth using an inverted microscope. The MEC endpoint was the lowest drug concentration that uniformly shortened the hyphae formation. Two independent observers read each well. If there was more than a 1 dilution difference in interpretation a third observer was used.

Mice. C57BL/6J (B6) female mice were purchased from Jackson Laboratory at 8 weeks of age and infected one week after arrival.

Infections and treatment. Standard BSL3 precautions were followed for all *in vivo* work.

Mice were infected intranasally as previously described, housed in cages inside a HEPA-filtered glove box which was contained inside a biological safety hood [40]. Briefly, they were anesthetized with a mixture of ketamine and xylazine and then ~200 spores (arthroconidia), suspended in 20ul sterile saline, were slowly dropped into their nares. After they recovered from the anesthesia, mice were placed 3 or 4 per cage in a HEPA-filtered glove box inside our BSL3 facility and allowed free access to food and water. Treatment by oral gavage while the mice were inside of the biological safety hood was initiated 7 days post infection and continued for 5 days. Fluconazole was administered orally as an aqueous solution at a dose of 25 mg/kg twice daily, and APX001 was diluted in 5% glucose and dosed orally at 50 mg/kg twice a day for 10 days in the first experiment. Mice were sacrificed one day after the last dose. In all subsequent experiments, treatment was initiated 7 days of infection using a regimen of 50 mg/kg of ABT by oral gavage followed 2 h later by oral gavage of 26 mg/kg of an APX prodrug.

Treatments continued for 5 days with control mice receiving 50 mg/kg ABT followed by buffer. One day after treatment ended (Day 13 post infection) mice were sacrificed for quantitative culturing of lungs and spleens, as previously described (40). The infection and quantitation of –CFU with APX001 was repeated three times with some minor variations in dosing, but a similar outcome. Fluconazole was only tested once but the results were consistent with published literature (REF).

Histology. On the last day of treatment mouse lungs were removed *en block* and then inflated through the trachea with glutaraldehyde. The lungs were then fixed overnight in glutaraldehyde and stained with periodic acid Schiff (PAS) by standard methods. PAS stains polysaccharides.

Statistics. Colony counts were $\log_{10}$ transformed and geometric means ±1 SEM CFU/organ were calculated, and two groups were compared using unpaired t test (GraphPad Prism 7.01, San Diego, CA). If there were greater than two groups, the difference in the means of treated and control groups were compared using Dunnett's ANOVA test. Kaplan-Meir survival curves were compared by log rank (Prism 7.01). A *P* value of ≤0.05 is considered statistically significant.

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Table 1. *In vitro* susceptibility profiles

	MEC (µg/ml)	MIC (µg/ml)*			
Strain	**APX001A**	**APX001A**	**FLC**	**AMB**	**POS**
C. immitis RS	0.002-0.004	8	>16	0.125	0.06-0.125
C. posadasii C735	0.004	0.03	>16	0.25	0.06-0.125
C. posadasii Silvera	0.008	8	>16	0.25	0.03

*MIC value was read at 100% inhibition

Abbreviations: FLC, fluconazole; AMB, amphotericin B; POS, posaconazole

Table 2. Activity of Gwt1 Inhibitors vs *C. immitis* and *C. posadasii*

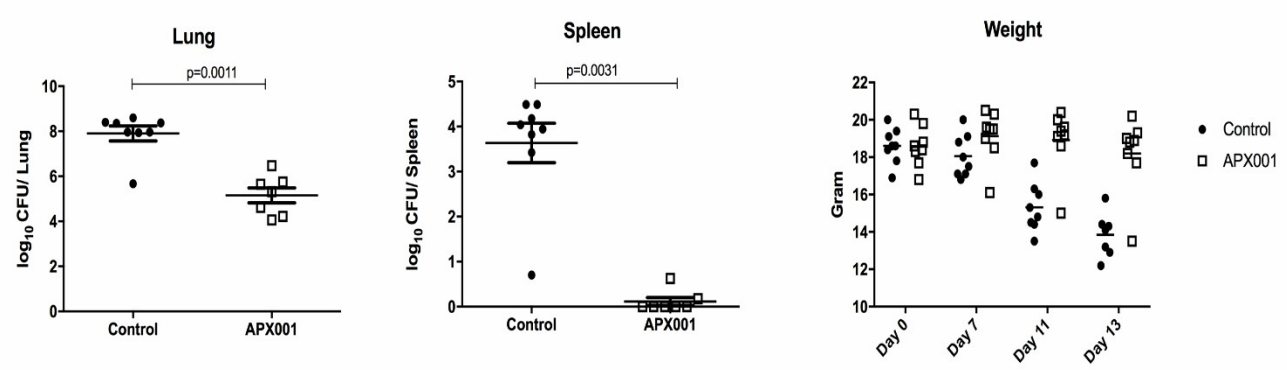
Strain	Source	MEC (µg/ml)			MIC (µg/ml)
		APX001A	APX2020	APX2041	POS
C. immitis RS	Lab	0.002-0.004[a]	0.002-0.004	0.002-0.004	0.06-0.125
C. immitis B2358	CDC	0.004	0.004	0.000125	0.016
C. immitis F40	Clinical	0.004	0.002	0.001	0.125
C. immitis F1	Clinical	0.002	0.001	0.001	0.125
C. immitis UCSD2	Clinical	0.001	0.001	0.00025	0.125
C. posadasii F6	Clinical	0.016	0.004	0.001	0.125
C. posadasii Silvera	Lab	0.008	0.008	0.004	0.03
C. posadasii F5	Clinical	0.008	0.004	0.001	0.016
C. posadasii C735	Lab	0.004	0.002	0.002	0.06-0.125
C. posadasii D2A	Clinical	0.004	0.002	0.001	0.03
GEOMEAN		0.004	0.002	0.001	0.054
MEC_{90}		0.008	0.004	0.002	0.125

a. The lower value of the susceptibility range was used to calculate the GEOMEAN and MEC_{90} values.

Fig. 1. Structures of Gwt1 inhibitors

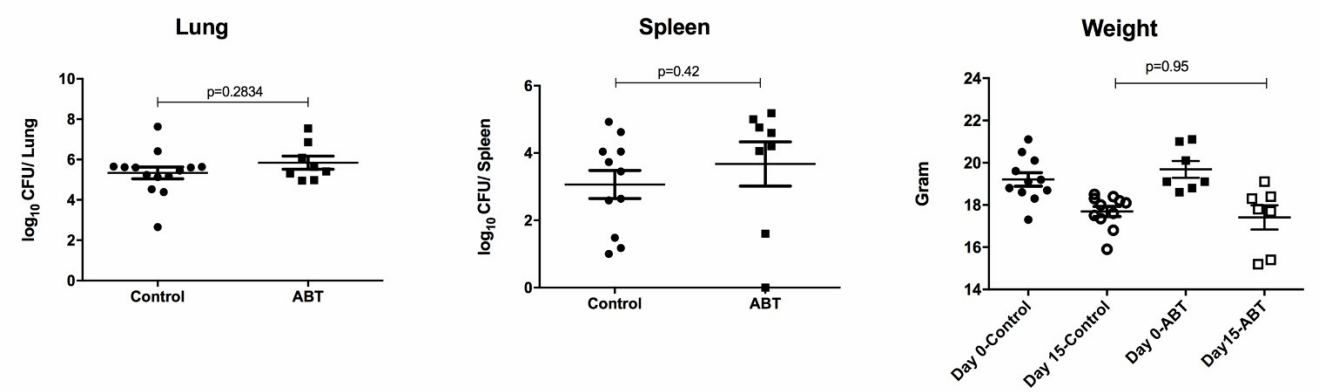
Compound	Structure	Prodrug
APX001A		APX001
APX2020		APX2097
APX2039		APX2096
APX2041		APX2104

Fig. 2. Efficacy of APX001 in a murine model of coccidioidomycosis



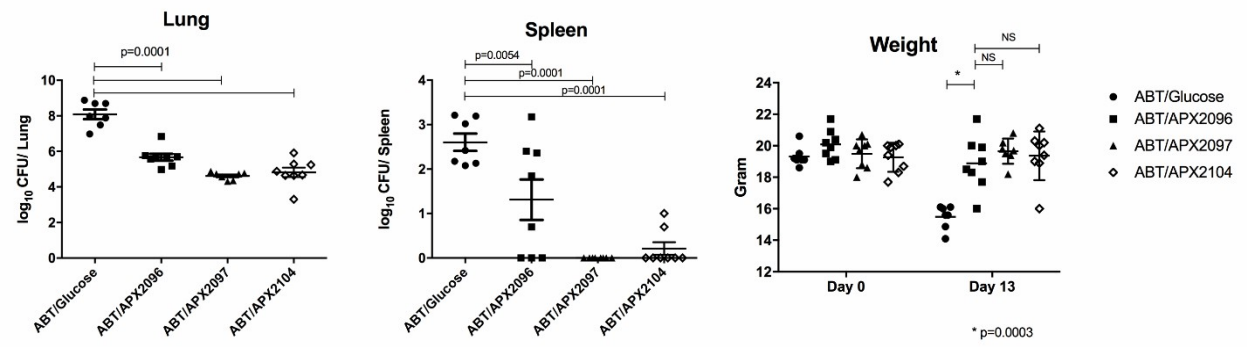
Mice were infected intranasally with *C. immitis* RS arthroconidia and 50 mg/kg of APX001 was administered twice daily for 5 days beginning 7 days post infection. Mice were sacrificed on Day 13, one day after the last day of treatment, and colony counts were assessed from lung and spleen. Each symbol represents one mouse. The horizontal lines show the geometric mean and SEM of lung and spleen colony counts (CFU). Horizontal lines in the weight figure correspond to the calculated mean weight. The difference in mean weight of treated and control mice on Days 11 and 13 were analyzed by two way ANOVA (GraphPad Prism) and were highly significant (P=<0.001).

Fig. 3. 1-Aminobenzotriazole (ABT) alone has no antifungal effect in mice



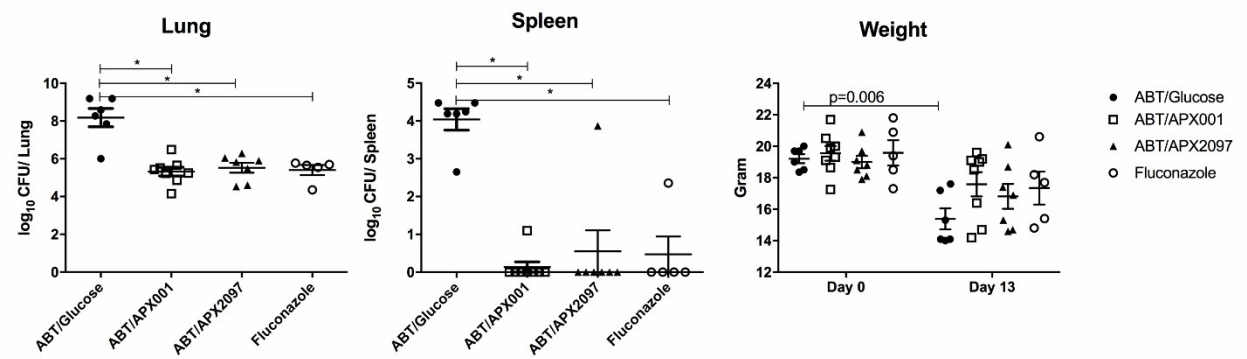
Infected mice were treated with a single daily dose of ABT for 5 days and sacrificed on Day 13, the day after the last ABT dose. Fungal colony counts were log transformed. Geometric mean ±1 SEM CFU/organ were calculated and compared using unpaired t test (GraphPad Prism 7.01, San Diego, CA). The mean weights ±1 SEM were calculated and there was no significant difference in the weights of untreated and ABT-treated mice on Day 15 after infection.

Fig. 4. Reduction in fungal burden upon treatment with three Gwt1 prodrugs in a mouse model of pulmonary coccidioidomycosis



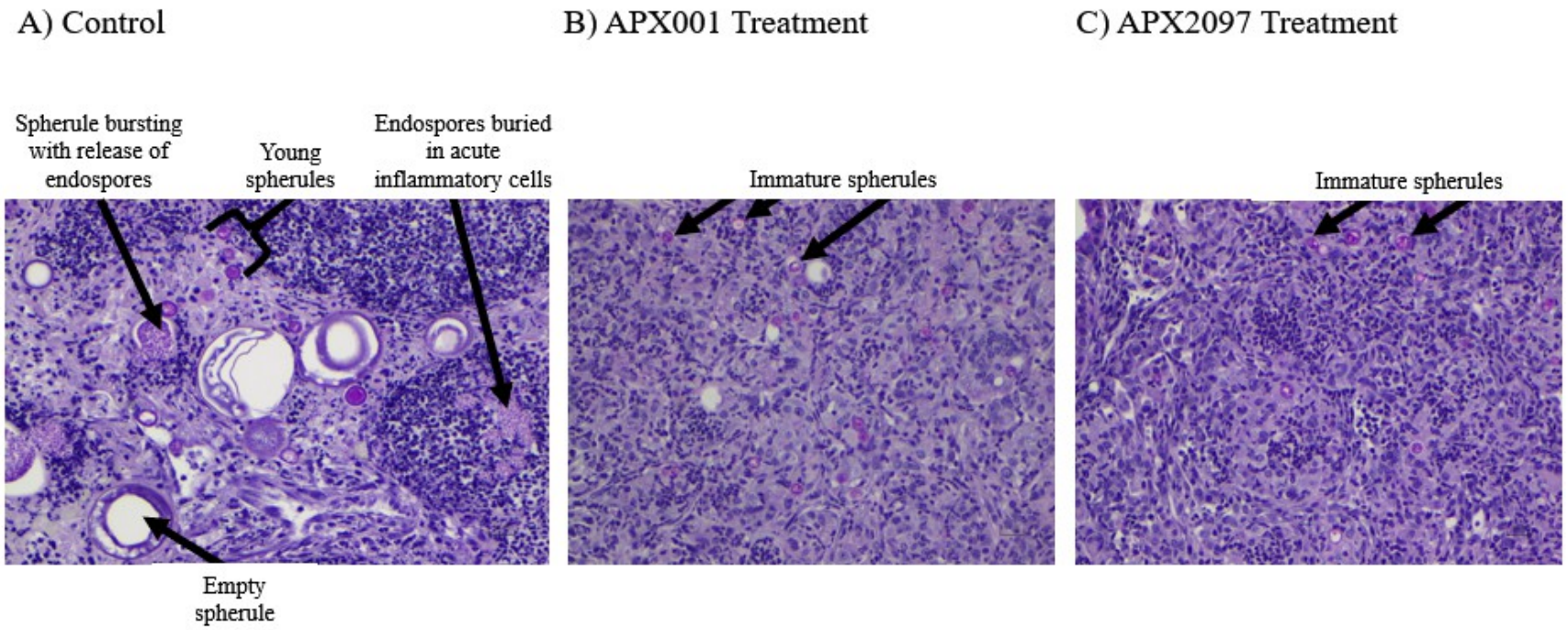
Mice were infected and treated as in **Fig. 2** except that mice were pre-treated with 50 mg/kg ABT by oral gavage 2 h prior to administration of APX prodrugs or buffer starting 7 days after infection. Mice were weighed at the start and conclusion of the experiment and were sacrificed one day after their last dose. After $\log_{10}$ transformation, geometric mean ±1 SEM CFU/organ were calculated and compared using unpaired t test (GraphPad Prism 7.01, San Diego, CA). If there were >2 groups the difference in the means of treated and control groups were compared using Dunnett's ANOVA test. A *P* value of ≤0.05 is considered statistically significant. * = P < 0.01 and NS = P >0.05 in the weight graph.

Fig. 5. Reduction in fungal burden upon treatment with APX001 and APX2097 in comparison with fluconazole



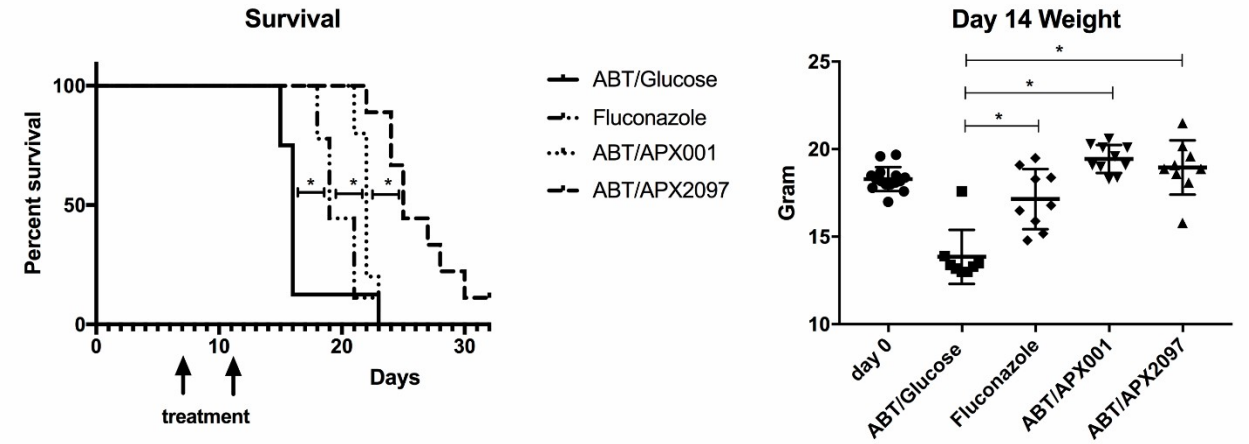
Mice were infected and treated with the ABT and APX drugs as in **Fig. 3.** Fluconazole was administered orally twice daily. Geometric mean ±1 SEM CFU/organ were calculated and compared using paired t test (GraphPad Prism 7.01, San Diego, CA). If there were >2 groups the difference in the means of treated and control groups were compared using Dunnett's ANOVA test. All the treatment groups had significant lower colony counts than the untreated control in lungs and spleen; * = P< 0.001. Only the untreated mice had a statistically significant eight loss on Day 13 after infection compared to their starting weight.

Fig. 6. Histological analysis of lung tissue sections in control vs APX001 and APX2097 treated mice.



Mice were infected with *C. immitis* RS as described in methods and then treated with 50 mg/kg ABT plus 26 mg/kg APX001 or APX2097 for 5 days. Control mice received only ABT. Lungs were removed a few hours after the last dose, fixed in glutaraldehyde, and then stained with PAS prior to microscopic examination (20X magnification). **A**) The control lungs showed many spherules in all stages of development and a myriad of endospores from ruptured spherules, surrounded by acute and chronic inflammatory cells. **B**) APX001 treated mice had many small, immature spherules that were primarily inside macrophages. There were no fully-grown spherules and few if any endospores. **C**) The lungs of APX2097 treated mice had a similar appearance to lungs of APX001 treated mice.

Fig. 7. Comparison of Kaplan-Meir survival curves and end of treatment weight of mice treated with APX001, APX2097, or fluconazole compared to untreated controls.



Mice were infected and treated as described in **Fig. 3**. The arrows show the days of treatment. Kaplan-Meir survival curves were compared by log rank (GraphPad Prism 7.01). All three treatment groups survived significantly longer than the control mice. Differences between the three treatment groups was also significant. Mean body weights of the three treatment groups and the untreated control on Day 14 post treatment were compared by ANOVA (Tukey's multiple comparisons (GraphPad Prism 7.01). There were no significant differences in the weights of fluconazole and APX001 and APX2097 treated mice. * = $P<0.01$ for both graphs.