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Journal

Antimicrobial Agents and Chemotherapy, 64(5)

ISSN

0066-4804

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Publication Date

2020-04-21

DOI

10.1128/aac.02223-19

Peer reviewed

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EXPERIMENTAL THERAPEUTICS

The Antifungal Drug Isavuconazole Is both Amebicidal and Cysticidal against *Acanthamoeba castellanii*

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ABSTRACT Current treatments for *Acanthamoeba* keratitis rely on a combination of chlorhexidine gluconate, propamidine isethionate, and polyhexamethylene biguanide. These disinfectants are nonspecific and inherently toxic, which limits their effectiveness. Furthermore, in 10% of cases, recurrent infection ensues due to the difficulty in killing both trophozoites and double-walled cysts. Therefore, development of efficient, safe, and target-specific drugs which are capable of preventing recurrent *Acanthamoeba* infection is a critical unmet need for averting blindness. Since both trophozoites and cysts contain specific sets of membrane sterols, we hypothesized that antifungal drugs targeting sterol 14-demethylase (CYP51), known as conazoles, would have deleterious effects on *A. castellanii* trophozoites and cysts. To test this hypothesis, we first performed a systematic screen of the FDA-approved conazoles against *A. castellanii* trophozoites using a bioluminescence-based viability assay adapted and optimized for *Acanthamoeba*. The most potent drugs were then evaluated against cysts. Isavuconazole and posaconazole demonstrated low nanomolar potency against trophozoites of three clinical strains of *A. castellanii*. Furthermore, isavuconazole killed trophozoites within 24 h and suppressed excystment of preformed *Acanthamoeba* cysts into trophozoites. The rapid action of isavuconazole was also evident from the morphological changes at nanomolar drug concentrations causing rounding of trophozoites within 24 h of exposure. Given that isavuconazole has an excellent safety profile, is well tolerated in humans, and blocks *A. castellanii* excystation, this opens an opportunity for the cost-effective repurposing of isavuconazole for the treatment of primary and recurring *Acanthamoeba* keratitis.

KEYWORDS *Acanthamoeba*, CYP51, conazole, cysticidal, drug screening, isavuconazole, keratitis

Acanthamoeba castellanii is a free-living ameba. It has been encountered in and isolated from various environmental sources, such as soil, dust, atmosphere, and water (1, 2). Its life cycle consists of a motile, feeding, and replicative ameboid stage (trophozoite) and a dormant cyst stage that is resistant to suboptimal environmental conditions (3).

A. castellanii may cause skin and brain infections, but is typically associated with *Acanthamoeba* keratitis, a painful, severe infection of the cornea that can result in blindness or visual impairment (4). While *Acanthamoeba* keratitis is rare, it is an emerging disease that has steadily increased in incidence over the past several decades (5, 6).

The most common routes of *Acanthamoeba* infection are through improperly cleaned contact lenses or corneal trauma (7). Once the trophozoites enter the eye, they invade the corneal epithelium and surrounding stroma (7, 8). The infection causes the rapid depletion of corneal keratocytes (8, 9). The infection may then trigger severe inflammation of the conjunctiva, cornea, episclera, and sclera (8, 9). In response,

Citation Shing B, Singh S, Podust LM, McKerrow JH, Debnath A. 2020. The antifungal drug isavuconazole is both amebicidal and cysticidal against *Acanthamoeba castellanii*. Antimicrob Agents Chemother 64:e02223-19. https://doi.org/10.1128/AAC.02223-19.

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Received 4 November 2019
Returned for modification 6 January 2020
Accepted 17 February 2020
Accepted manuscript posted online 24 February 2020
Published 21 April 2020

neutrophils and macrophages will infiltrate the cornea (7, 9). Infiltration of neutrophils leads to further necrosis in the cornea (8).

If the infection is not cleared through medical intervention, the trophozoites can spread to the retina and cause chorioretinitis (10–12). The most aggressive and severe cases of *Acanthamoeba* keratitis require corneal grafts or surgical removal of the eye (13). *Acanthamoeba* keratitis can occur in immunocompetent patients, and clearance of the infection does not provide sterilizing immunity (8).

In the United States, the majority (>80%) of *Acanthamoeba* keratitis cases involve contact lens wearers (14, 15). As of 2017, the contact lens industry generates $11.5 billion annually and serves approximately 45 million contact lens wearers in the United States. Nevertheless, there has been minimal interest in identifying and developing new drugs to effectively treat or prevent *Acanthamoeba* keratitis (16–18).

Current *Acanthamoeba* keratitis treatments rely on diamidines, biguanides, and antifungal azole derivatives (conazoles) to kill the trophozoites (7). Treatment typically consists of a combination of chlorhexidine gluconate, propamidine isethionate, and polyhexamethylene biguanide (PHMB) (6). While PHMB and chlorhexidine are effective at killing *Acanthamoeba* trophozoites, these drugs are aggressive and cannot be tolerated at high doses, which limits their effectiveness (19). Antifungal azole derivatives, such as clotrimazole, miconazole, ketoconazole, and itraconazole, have also been used clinically with limited efficacy to treat *Acanthamoeba* keratitis (8). Overall, in approximately 10% of all *Acanthamoeba* keratitis cases, recurrent infection ensues due to the difficulty of killing both *Acanthamoeba* trophozoites and cysts (16).

While *A. castellanii* has proven to be a difficult pathogen to treat effectively, previous work, including clinical studies, has suggested sterol biosynthesis could be targeted to inhibit *Acanthamoeba* trophozoites (20, 21). In *Acanthamoeba* trophozoites, ergosterol is indispensable and the major biosynthetic pathway for producing ergosterol is through the conversion of cycloartenol (22, 23). Sterol 14-demethylase (CYP51) is an essential enzyme in ergosterol biosynthesis both in fungi and protozoa. *A. castellanii* encodes a CYP51 with sequence identity of 31 to 35% to fungal CYP51 (20). Previous studies demonstrated that inhibition of CYP51 led to reduced growth of *A. castellanii* trophozoites and induced encystment that produced nonviable cysts (20, 22, 24).

Antifungal azole derivatives known as conazoles are FDA-approved CYP51 inhibitors that include itraconazole, voriconazole, posaconazole, fluconazole, ketoconazole, clotrimazole, isavuconazole, and miconazole (25, 26). These drugs inhibit fungal CYP51 to prevent the conversion of lanosterol to ergosterol, which causes the rapid depletion of ergosterol and the accumulation of ergosterol precursors and nonphysiological end products (27, 28). Since ergosterol is a major component of fungal membranes, depleting this sterol causes leakage of cell membranes, leading to cell death (28). While azole derivatives have primarily been approved for treating fungal infections, some of these drugs have also been evaluated for treating *Acanthamoeba* keratitis, but with limited success.

Current methods used for identifying amebicidal compounds against *Acanthamoeba* are labor-intensive and rely upon microscopy and traditional cell-counting methods using hemocytometers and staining agents (29–32). Until recently, most efforts to identify new anti-*Acanthamoeba* compounds via whole parasite screening have focused on the development of screening methodology and its validation by reference drugs, such as chlorhexidine (32). In this study, we optimized a commercially available bioluminescence-based viability assay for high-throughput screening of compounds against *Acanthamoeba* trophozoites. Using this assay, we systematically assessed the FDA-approved conazoles and identified isavuconazole as the most potent target-specific anti-*Acanthamoeba* agent. As an added benefit, isavuconazole was effective against both *A. castellanii* trophozoites and cysts.

RESULTS

Viability assay for *A. castellanii* trophozoites. Assays commonly used to assess amebicidal activity of compounds against *Acanthamoeba* are labor-intensive and not

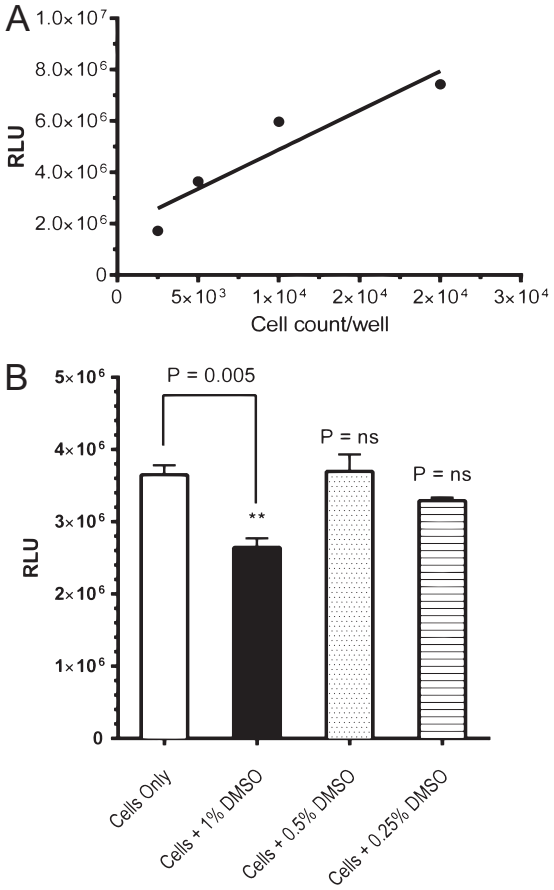

FIG 1 Viability assays for *A. castellanii* trophozoites. (A) Correlation between the number of viable *A. castellanii* trophozoites and ATP-bioluminescence in a 96-well microtiter plate. Values plotted are the means and standard deviations of triplicate wells. The line represents a regression curve for plotted data. RLU, relative light unit. (B) Tolerability of DMSO by *A. castellanii* trophozoites. Trophozoites (5×10^3) were either treated with different concentrations of DMSO (%) or left untreated in a 96-well plate format. ATP-bioluminescence was measured after 48 h. Values plotted are the means and standard deviations of triplicate wells. ns, not significant, with $P > 0.05$ by Student's *t* test compared to untreated *A. castellanii* trophozoites.

easily amenable to high-throughput compound screening (29–32). To accelerate anti-*Acanthamoeba* drug discovery, we adopted the CellTiter-Glo luciferase-based assay (Promega) that is based on the correlation between the number of live microorganisms and the ATP level. In *Acanthamoeba*, the relationship between the number of trophozoites seeded into 96-well plates and luminescence from the CellTiter-Glo assay showed a strong linear correlation ($R^2 = 0.88$) (Fig. 1A). A total of 5×10^3 trophozoites per well were used in subsequent experiments. When various amounts of dimethyl sulfoxide (DMSO) were added to a culture of 5×10^3 trophozoites in the 96-well microtiter plate format, trophozoites readily tolerated up to 0.5% (vol/vol) DMSO with no statistically significant degradation of growth rate (Fig. 1B). At 1% DMSO (vol/vol) treatment, trophozoites showed significant growth inhibition (Fig. 1B).

***In vitro* activity of CYP51 inhibitors against *A. castellanii* trophozoites.** Given that activity of some azole antifungal drugs against *A. castellanii* has been demonstrated by different laboratories (20, 22, 33), we took advantage of the newly developed microtiter plate ATP bioluminescence-based assay to systematically evaluate eight FDA-approved conazoles, including the latest addition to the armamentarium of the antifungal drugs, isavuconazole. For the drug screen, we selected the *A. castellanii* Ma strain, representing the T4 genotype, because nearly all reported *Acanthamoeba* keratitis infections are associated with the T4 genotype (34). The drugs demonstrated

TABLE 1 EC_{50} values of different azoles, chlorhexidine, and PHMB against *A. castellanii* trophozoites

Compound	Strain	EC_{50} (μM)		
		Mean	95% lower CL[b]	95% upper CL[b]
Azoles				
Fluconazole	Ma	3% at 50 μM[a]		
Itraconazole	Ma	54% at 50 μM[a]		
Miconazole	Ma	6.9	5.8	8.3
Ketoconazole	Ma	1.8	1.4	2.0
Voriconazole	Ma	0.6	0.4	0.8
Clotrimazole	Ma	0.2	0.1	0.2
Posaconazole	Ma	0.045	0.039	0.051
	MEEI 0184	0.003	0.002	0.004
	CDC:V240	0.065	0.054	0.079
Isavuconazole	Ma	0.005	0.004	0.006
	MEEI 0184	0.026	0.023	0.028
	CDC:V240	<0.001	<0.001	0.001
Standards of care				
Chlorhexidine	Ma	1.7	1.4	1.9
	MEEI 0184	1.0	0.9	1.1
	CDC:V240	1.1	1.0	1.2
PHMB	Ma	7.2	6.6	8.0
	MEEI 0184	4.6	3.0	7.1
	CDC:V240	11.8	10.5	13.4

[a]For two azoles, fluconazole and itraconazole, the value given is the percentage of growth inhibition at the highest tested concentration, as the mean EC_{50} value was not identified.
[b]CL, confidence limit.

activity against *A. castellanii* in a range of 3% growth inhibition at 50 μM for fluconazole to a 50% effective concentration (EC_{50}) of 5 nM for isavuconazole (Table 1). Notably, the *in vitro* potency of isavuconazole, voriconazole, clotrimazole, posaconazole, and ketoconazole exceeded that of the current drugs chlorhexidine and PHMB. Isavuconazole, posaconazole, clotrimazole, and voriconazole were an order of magnitude more potent than chlorhexidine, while ketoconazole was equipotent to chlorhexidine. Since isavuconazole and posaconazole demonstrated a low nanomolar potency against the *A. castellanii* Ma strain, their potencies against other *A. castellanii* clinical strains of T4 genotype were also evaluated. In the *A. castellanii* CDC:V240 and *A. castellanii* MEEI 0184 strains, the 48-h EC_{50} values of isavuconazole were determined to be 0.9 nM and 25.7 nM, respectively (Fig. 2B and C), which are comparable in magnitude to the *A. castellanii* Ma strain's 48-h EC_{50} of 4.6 nM (Fig. 2A). Posaconazole was also assayed against *A. castellanii* CDC:V240 and *A. castellanii* MEEI 0184 and the 48-h EC_{50} values were determined to be 65.3 nM and 3.0 nM, respectively (Fig. 2E and F), which are comparable to the *A. castellanii* Ma strain's 48-h EC_{50} of 44.5 nM (Fig. 2D). These growth inhibition curves suggest that the two most potent drugs, isavuconazole and posaconazole, are broadly efficacious against *A. castellanii* trophozoites (Table 1).

Growth inhibition as a function of time. To assess how quickly isavuconazole and posaconazole kill trophozoites, growth inhibition of *A. castellanii* Ma strain trophozoites was measured at 16, 24, 36, and 48 h of drug exposure. Trophozoites were exposed to isavuconazole or posaconazole serially diluted from 50 μM to 5.96 pM in DMSO. The resulting growth inhibition curves (Fig. 3) show that isavuconazole and posaconazole have similar inhibitory effects. Both drugs reached ~50% inhibition at 24 h of exposure and ~90% at 36 h of exposure. The effect of both drugs maximized at 48 h, when the isavuconazole potency (EC_{50} of 0.005 μM) exceeded the posaconazole potency (EC_{50} of 0.04 μM) by ~10-fold (Fig. 3). Although chlorhexidine (EC_{50} of 1.7 μM) and PHMB (EC_{50} of 7.3 μM) were faster-acting drugs than conazoles, with measurable inhibition as early as 16 h of exposure (Fig. 3), target-specific posaconazole and isavuconazole demonstrated potency 40-fold and 300-fold, respectively, higher than the current standard-of-care chlorhexidine.

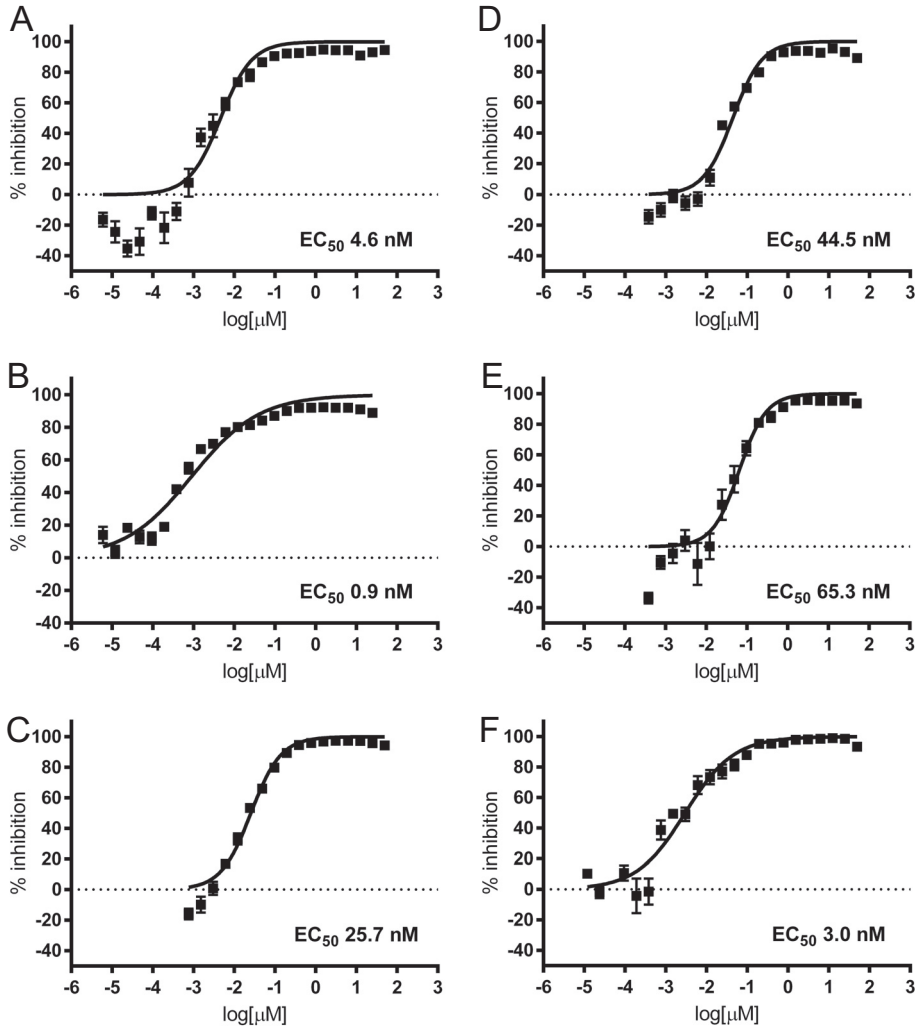

FIG 2 Concentration-dependent inhibition of growth of three strains of *A. castellanii* trophozoites by isavuconazole and posaconazole. Growth inhibition curve comparisons between *A. castellanii* Ma, *A. castellanii* CDC:V240, and *A. castellanii* MEEI 0184 strains at 48 h. (A) *A. castellanii* Ma treated with isavuconazole; (B) *A. castellanii* CDC:V240 treated with isavuconazole; (C) *A. castellanii* MEEI 0184 treated with isavuconazole; (D) *A. castellanii* Ma treated with posaconazole; (E) *A. castellanii* CDC:V240 treated with posaconazole; and (F) *A. castellanii* MEEI 0184 treated with posaconazole. Data points represent mean percentage growth inhibition and standard error of mean (SEM) of different concentrations of isavuconazole and posaconazole. EC_{50} curves were generated from mean values of percentage growth inhibition ± SEM of isavuconazole and posaconazole against *A. castellanii*.

Effect of isavuconazole on trophozoite morphology and viability. Since isavuconazole was the most potent anti-*Acanthamoeba* azole identified in this study, we assessed the effect of isavuconazole on trophozoite viability and morphology. *A. castellanii* Ma strain trophozoites were treated with 45 nM (~10× EC_{50} concentration) of isavuconazole and the effect elicited by this concentration of isavuconazole was compared with the effect elicited by 16.6 μM chlorhexidine (~10× EC_{50} concentration).

Trophozoites treated with 0.5% DMSO displayed normal morphology and growth. The trophozoites displayed membrane integrity at 24, 36, and 48 h. Each trophozoite displayed clearly visible food vacuoles and a large nucleus containing the nucleolus. There was no evidence of DMSO growth inhibition at 24, 36, or 48 h (Fig. 4A). Treatment with 16.6 μM chlorhexidine led to cell death as early as 24 h postexposure (Fig. 4B). No cell proliferation was observed from 24 to 48 h. Instead, significant amounts of cell debris and membrane components were observed in the medium of chlorhexidine-treated trophozoites, which is consistent with cell lysis. Chlorhexidine-treated tropho-

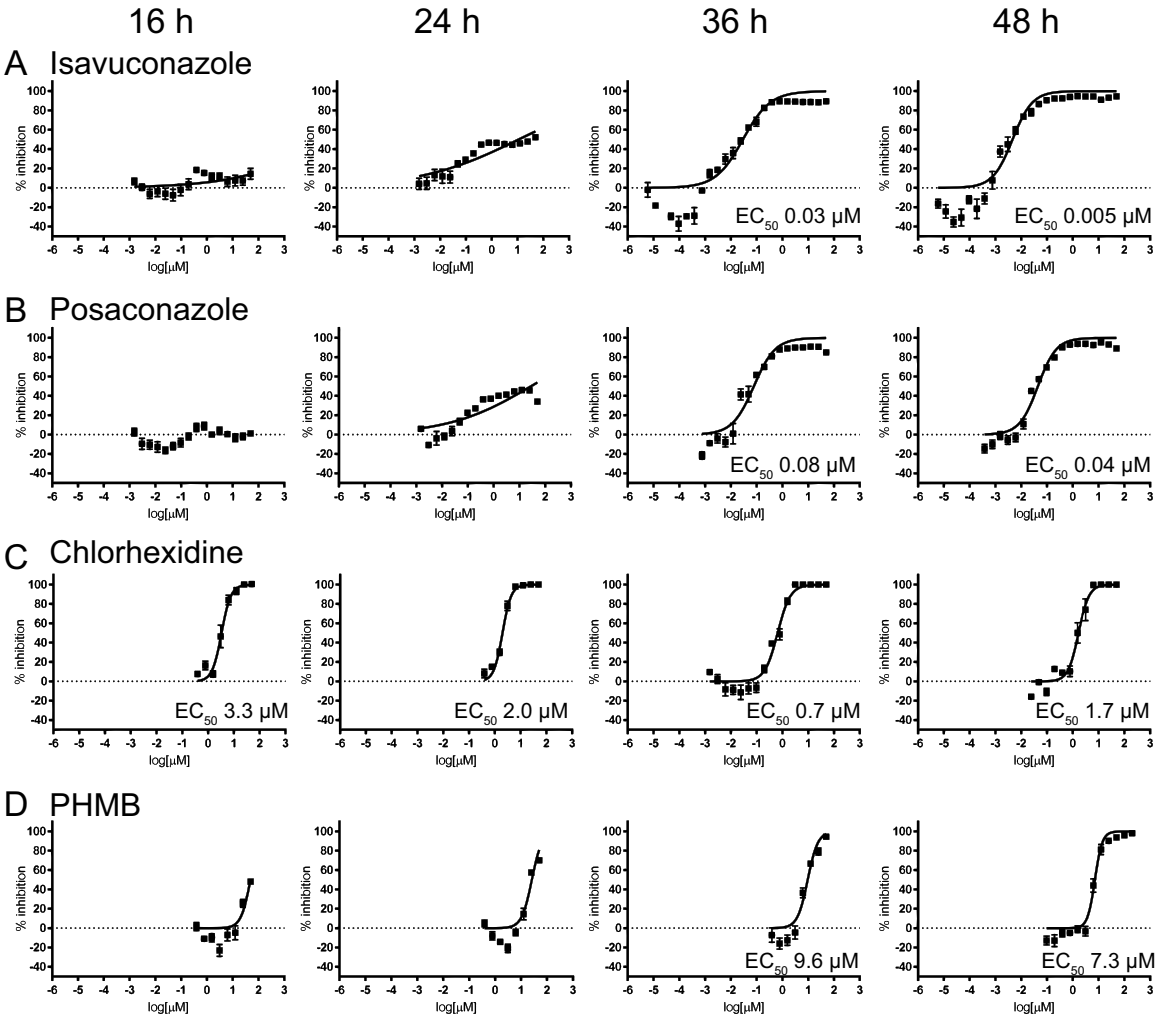

FIG 3 Growth inhibition curves of *A. castellanii* Ma strain at different time points. Growth inhibition curves of trophozoites treated with isavuconazole (A), posaconazole (B), chlorhexidine (C), and PHMB (D) at 16, 24, 36, and 48 h. Data points represent mean percentage growth inhibition and standard error of the mean (SEM) of different concentrations of compounds.

zoites displayed abnormal morphology characterized by a significant increase in cellular granularity and the disappearance of food vacuoles and nucleolus structure. Treatment with isavuconazole at 45 nM likewise resulted in significant growth inhibition and cell death (Fig. 4C). At 24 h, isavuconazole-treated trophozoites began showing signs of cellular rounding compared to the DMSO-treated trophozoites. At 36 and 48 h, the trophozoites completely rounded and detached from the bottom of the culture plate. There was also an appreciable increase in cellular granularity and no significant cellular proliferation between 36 and 48 h (Fig. 4C). Furthermore, the medium of isavuconazole-treated trophozoites displayed more cellular debris and membrane components than that of 16.6 μM chlorhexidine.

Evaluation of cysticidal activity. The cysticidal activity of isavuconazole and posaconazole was evaluated using cysts of the *A. castellanii* Ma strain. Preformed cysts were treated with various concentrations (200, 150, 100, 90, 80, 70, 60, 50, 40, and 30 μM) of isavuconazole or posaconazole. Chlorhexidine (0.02% [wt/vol], equivalent to 395.7 μM) and PHMB (equivalent to 1079.5 μM) were used as positive controls, while 0.5% DMSO was used as a negative control.

Isavuconazole was cysticidal at 70 μM (Fig. 5A). At isavuconazole concentrations below 70 μM (Fig. S1A and S1B in the supplemental materials), various levels of delayed excystation were observed. Excystation was only apparent at day four. Following

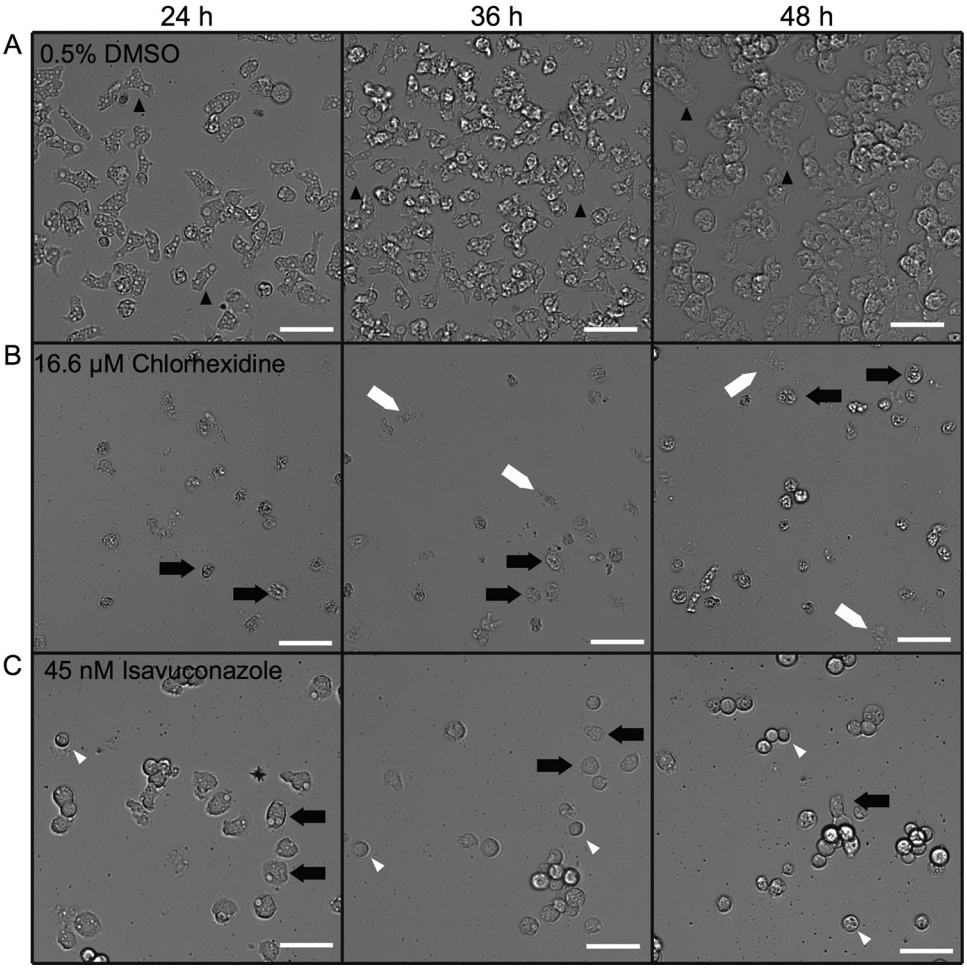

FIG 4 Effect of isavuconazole and chlorhexidine on the morphology and viability of *A. castellanii* Ma trophozoites. Trophozoites were treated with 0.5% DMSO (A), 16.6 μM chlorhexidine (10× EC_{50} value) (B), and 45 nM isavuconazole (10× EC_{50} value) (C). Trophozoites were imaged at 24, 36, and 48 h. Black arrowheads indicate healthy and proliferating trophozoites. White arrowheads indicate stressed and rounded trophozoites. Black arrows indicate shrunken trophozoites. White arrows indicate lysed cells. Magnification, 200×; bars, 50 μm.

treatment with 30 μM isavuconazole, cultures became confluent with trophozoites by day six (Fig. S1B).

Remarkably, treatment with posaconazole did not prevent excystation. The highest concentrations of posaconazole tested (200 and 150 μM) still showed signs of excystation and became confluent with healthy trophozoites by day six (Fig. 5B, Fig. S1C).

Treatment with chlorhexidine or PHMB (0.02% [wt/vol]) prevented excystation (Fig. 5C and D), suggesting that chlorhexidine and PHMB killed the cysts. By day four, the DMSO-treated cysts displayed normal excystation (Fig. 5E) with no evidence of any remaining cysts and became confluent with healthy trophozoites.

DISCUSSION

In this study, a luciferase-based viability assay was adopted and optimized for *Acanthamoeba* to accelerate screening for amebicidal compounds. This assay was previously used with a number of other pathogens, including trypanosomes, *Entamoeba*, *Giardia*, and *Naegleria* (35–41). It represents a clear improvement over current *Acanthamoeba* methods that require a tedious liquid and cell transfer and prolonged read-outs. It also reduces the cost of reagents and hours of labor required to screen large compound libraries. The utility of the newly developed luciferase-based method was validated for *A. castellanii* trophozoites by systematically assessing a set of drugs,

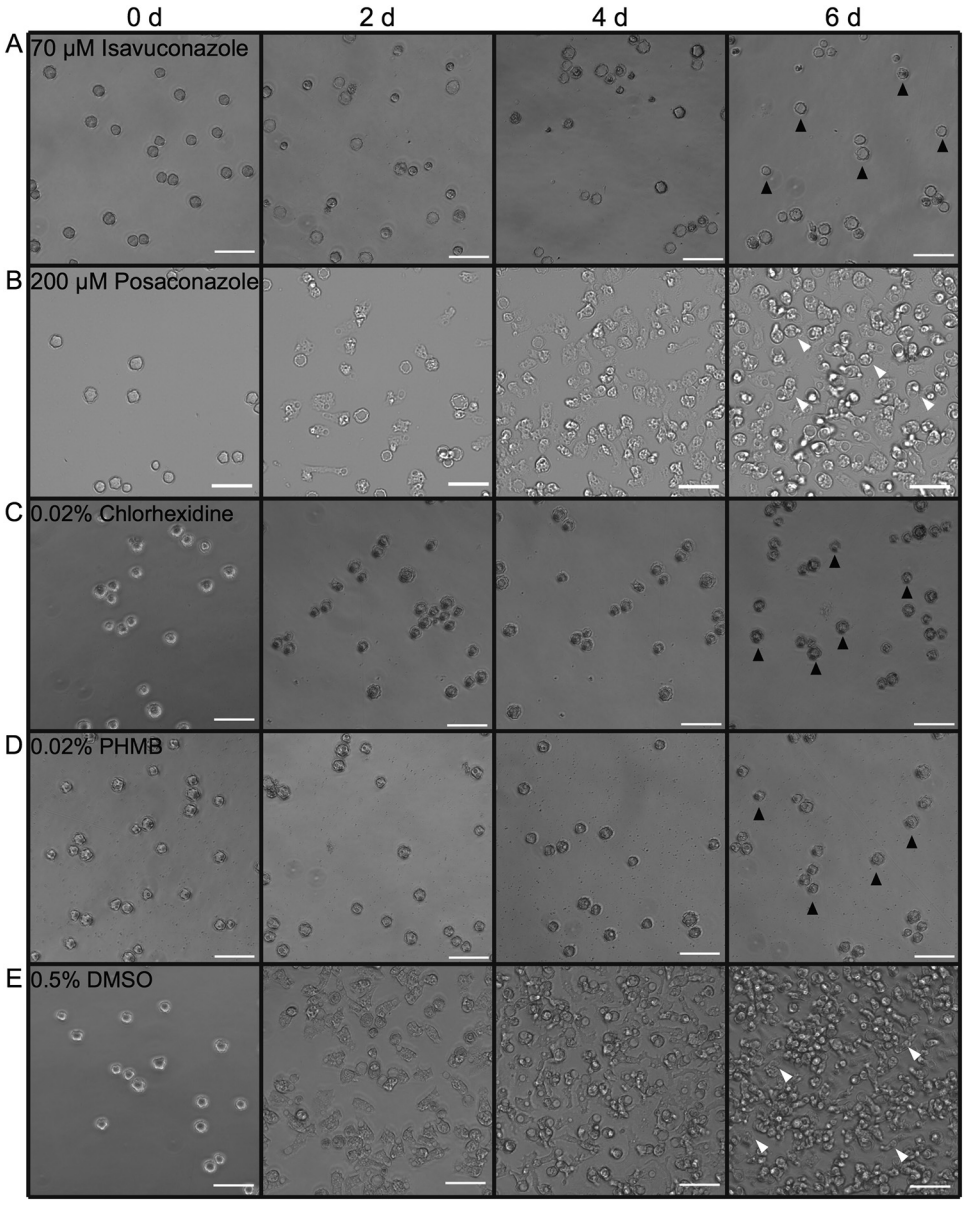

FIG 5 Effect of isavuconazole on the morphology of *A. castellanii* Ma cysts. Cysts were treated with (A) 70 μM isavuconazole (A), 200 μM posaconazole (B), 0.02% (wt/vol) chlorhexidine (C), 0.02% (wt/vol) PHMB (D), and 0.5% (vol/vol) DMSO (E) for 48 h and switched to PYG medium 48 h posttreatment. Cyst morphology and excystation were monitored over the course of a week after exchanging the old medium with fresh PYG medium. Black arrowheads indicate unviable cysts, white arrowheads indicate trophozoites that excysted. Magnification, 200×; bars, 50 μm.

known as conazoles or antifungal azoles, which target the 14-demethylation step in sterol biosynthesis catalyzed by CYP51.

Conazoles were developed as antifungal agents, but their therapeutic potential has also been demonstrated in kinetoplastids (21) and *Naegleria* (23). Select azole antifungals tested in previous studies against *A. castellanii* exhibited potencies comparable to that of chlorhexidine (20, 22, 42), which encouraged us to systematically assess this class of drugs for *A. castellanii* growth inhibition. We identified two azoles, posaconazole and isavuconazole, to be about 40-fold and 300-fold more potent against *A. castellanii* Ma strain than the current standard-of-care chlorhexidine. While posaconazole was about 17- to 300-fold more potent than chlorhexidine against CDC:V240 and MEEI 0184 strains, isavuconazole exhibited about 40- to 1,000-fold more activity than

chlorhexidine against MEEI 0184 and CDC:V240 strains. Posaconazole was previously reported as amebicidal (43). However, isavuconazole, a newer broad-spectrum antifungal drug, is demonstrated here to be the most potent amebicidal agent among conazoles tested so far. Isavuconazole was approximately 10-fold and 65-fold more potent than posaconazole against Ma and CDC:V240 strains, respectively, and exhibited low nanomolar potency against three clinical strains of *A. castellanii*. Both isavuconazole and posaconazole were relatively fast-acting against *A. castellanii* trophozoites, with 50% growth inhibition achieved as early as 24 h postexposure. The rapid activity of isavuconazole was also evident from morphological studies, where nanomolar concentrations of isavuconazole promoted rounding of cells within 24 h of treatment and had effects comparable to low micromolar concentrations of chlorhexidine.

The inhibitory activity of isavuconazole and posaconazole against trophozoites led us to test their effectiveness against *A. castellanii* cysts. A sterol metabolome study of *A. castellanii* revealed that marked changes in sterol composition are associated with ameba differentiation (24). Stage-specific sterol profiling during the growth and encystment phases identified metabolic markers for viable and nonviable cysts. This previous metabolome study showed that only viable cysts can excyst into trophozoites (24). To determine if posaconazole and isavuconazole could suppress excystment of preformed *Acanthamoeba* cysts into trophozoites, we established an *Acanthamoeba* encystation assay in a 96-well format and treated mature cysts with different concentrations of drugs. Our microscopy-based assay found that treating mature cysts of the *A. castellanii* Ma strain for 48 h with 70 μM (30.6 μg/ml) of isavuconazole prevented excystation. Treatment of cysts of the same strain with 200 μM posaconazole did not prevent excystation. This is in contrast to a reported study where the minimal cysticidal concentration of posaconazole was found to be 57 μM or 114 μM against two different strains of *A. castellanii* (44). It is not clear if the differences in the cysticidal activity of posaconazole are due to the differences in the strains used in the excystation studies or due to the differences in the methods used to demonstrate the cysticidal activity of posaconazole.

Isavuconazonium sulfate, a water-soluble isavuconazole prodrug, is the most recently developed antifungal triazole drug, approved in 2015 by the FDA for treating adults with invasive aspergillosis or invasive mucormycosis. Isavuconazole is given either in an intravenous (IV) or oral formulation at 200 mg once daily, following a loading dose of 200 mg every 8 h for the first 48 h (45). The drug is readily absorbed when administered orally, with a bioavailability of 98% (46). It has a half-life of 130 h (45) and a large volume of distribution (400 to 500 liters) (47). A phase 3 clinical trial that assessed the efficacy and safety of isavuconazole and voriconazole in patients with invasive aspergillosis found that isavuconazole was well tolerated with significantly fewer drug-related adverse events of the skin, eye, and hepatobiliary systems than voriconazole (48).

Although isavuconazole is only currently available in IV and oral formulations and *Acanthamoeba* keratitis treatment conventionally requires topical administration, CYP51 inhibitors can meet the demand for development of topical anti-*Acanthamoeba* keratitis agents. Ophthalmic formulation has been developed to topically administer another antifungal azole drug, econazole, which has poor aqueous solubility (49).

An economic model to determine the costs and cost-effectiveness of isavuconazole versus voriconazole in hospitalized patients with invasive aspergillosis suggested that isavuconazole was a cost-effective option (50). Based on the wholesale acquisition costs from ReadyPrice (Thomson), the price of 372 mg of isavuconazonium sulfate (equivalent to 200 mg of isavuconazole) was $238.50 and $70.00 for the IV and oral formulations, respectively (50). Considering the shorter treatment schedule for *Acanthamoeba* keratitis, treatment with isavuconazole may be cost-effective.

Future studies will involve the development of an ophthalmic formulation and testing the efficacy of isavuconazole in an animal model of *Acanthamoeba* keratitis. Based on its excellent safety profile, isavuconazole presents an opportunity to cost-

effectively repurpose this drug for the treatment of primary and recurring *Acanthamoeba* keratitis.

MATERIALS AND METHODS

***A. castellanii* strains and cultures.** The *A. castellanii* reference strain Ma was acquired from the American Type Culture Collection (number 50370). The *A. castellanii* CDC:V240 strain of the T4 genotype was acquired from the Centers for Disease Control and Prevention (CDC) and the *A. castellanii* MEEI 0184 strain of the T4 genotype (51) was obtained from Tufts University. *A. castellanii* trophozoites were cultured and maintained at 28°C and 5% CO_2 in peptone yeast glucose (PYG) medium supplemented with 1% penicillin-streptomycin (52, 53).

Cyst generation. *A. castellanii* (Ma strain) encystment was induced by culturing trophozoites in a modified Page's ameba saline encystation medium (95 mM NaCl; 5 mM KCl; 8 mM $MgSO_4$; 0.4 mM $CaCl_2$; 1 mM $NaHCO_3$; 20 mM Tris-HCl, pH 9.0) (54). *A. castellanii* trophozoites were collected by centrifugation at 200 × g for 5 min. The trophozoites were then washed in phosphate-buffered saline (PBS) twice before being resuspended in encystation medium. The cells were cultured in the encystation medium for at least 48 h to generate cysts prior to any experiments requiring *A. castellanii* cysts.

Viability assay for *A. castellanii* trophozoites. The assay was developed in sterile 96-well microtiter plates with exponentially grown, 48 h-old *A. castellanii* Ma strain trophozoites. ATP is an essential cofactor for biogenesis in *A. castellanii*, so a luciferase-based assay was used to validate the correlation between the number of viable trophozoites and their ATP levels. Trophozoites were counted and 2.5×10^3, 5×10^3, 10×10^3, or 20×10^3 trophozoites in 100 μl of PYG medium were seeded into the wells of 96-well microtiter plates under sterile conditions. Assay plates were incubated for 48 h at 28°C and 5% CO_2. At the end of the incubation, the plates were equilibrated to room temperature for 30 min. An aliquot of 25 μl of CellTiter-Glo luminescent cell viability assay solution (Promega) was added to each well. The microplates were shaken on a microplate orbital shaker (VWR) at 360 rpm for 10 min to facilitate cell lysis, and the plates were then incubated for an additional 10 min to stabilize the luminescent signal. The resulting ATP-bioluminescence of the trophozoites was measured by an EnVision 2104 Multilabel Reader (Perkin Elmer) at room temperature.

Once the number of viable trophozoites that would be used in the subsequent experiments was determined, trophozoites were then treated with various amounts of DMSO (ranging from 0% to 1%) to optimize the percentage of DMSO that *Acanthamoeba* could tolerate. Trophozoites were incubated at 28°C and 5% CO_2 for 48 h, and the ATP-bioluminescence was assayed at the end of the incubation.

***In vitro* activity of CYP51 inhibitors against *A. castellanii* trophozoites.** Azole stocks were prepared in DMSO at a concentration of 20 mM, while PHMB and chlorhexidine control drugs were dissolved in DMSO at a concentration of 10 mM. The conazoles and control drugs were serially diluted one to two parts 24 times in DMSO to generate solutions from 10 mM to 1.2 nM. The compounds were then added to 96-well Greiner Bio-One Cellstar white, flat bottom microplates. Aliquots of 0.5 μl of drugs were added to each well. *A. castellanii* trophozoites were counted, and 5×10^3 trophozoites in 99.5 μl of PYG media were added to each well.

After incubation for 48 h at 28°C and 5% CO_2, cell viability measurements were taken using the CellTiter-Glo luminescent cell viability assay on the EnVision 2104 Multilabel Reader. The data were analyzed on GraphPad Prism 6 to determine EC_{50} values and 95% confidence intervals.

Growth inhibition as a function of time. To determine the rate of killing, growth inhibition of the *A. castellanii* Ma strain trophozoites was measured for isavuconazole, posaconazole, chlorhexidine, and PHMB at 16 h, 24 h, 36 h, and 48 h of exposure to serially diluted drug concentrations ranging from 50 μM to 5.96 pM. The growth inhibition assay was done in triplicate in three independent experiments and the EC_{50} values of isavuconazole and posaconazole were determined at different time points by CellTiter-Glo luminescent cell viability assay.

Effect of isavuconazole on trophozoite morphology and viability. 5×10^3 trophozoites in 99.5 μl of PYG medium were plated onto clear 96-well flat bottom microplates and the trophozoites were treated with 0.5% DMSO, 16.6 μM of chlorhexidine, and/or 45 nM isavuconazole for 48 h. The effect of isavuconazole on cellular morphology was determined by imaging the trophozoites at 24, 36, and 48 h and compared to the effect of chlorhexidine. Images were acquired using the Molecular Devices ImageXpress Micro XLS and adjusted for brightness and contrast in ImageJ.

Evaluation of cysticidal activity. *A. castellanii* trophozoites were collected by centrifugation at 200 × g for 5 min. The trophozoites were then washed in PBS three times before resuspension in encystation medium. Aliquots of 5×10^3 trophozoites were added to each well of a 96-well plate and wells were filled to 100 μl with encystation medium. The cells were then incubated for 48 h prior to addition of compounds.

After 48 h, the encystation medium in each well was exchanged for encystation medium with 200, 150, 100, 90, 80, 70, 60, 50, 40, or 30 μM azole (isavuconazole or posaconazole). DMSO at 0.5% served as a negative control, while 0.02% (wt/vol) chlorhexidine and 0.02% (wt/vol) PHMB served as positive controls. The mature cysts were then incubated with compounds for an additional 48 h. At the end of 96 h, the encystation medium was washed three times with PBS before addition of 100 μl of PYG medium. *A. castellanii* cysts were then incubated in PYG media and imaged daily on a Zeiss Axio Vert.A1 microscope and Molecular Devices ImageXpress Micro XLS. After every additional 48 h, the medium was exchanged for 100 μl of fresh PYG medium. Images were adjusted for brightness and contrast in ImageJ.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 3.5 MB.

ACKNOWLEDGMENTS

We are grateful to Noorjahan Panjwani of Tufts University and Ibne K. Ali of the CDC for providing *A. castellanii* MEEI 0184 and CDC:V240 strains, respectively.

This work was partially supported by a UC San Diego Chancellor's Research Excellence Scholarship (to A.D.). A. D. was supported by grant 1KL2TR001444 from the NIH and grants R21AI133394 and R21AI141210 from the NIAID. L.M.P. was supported in part by a UCSD Academic Senate Research grant.

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