



Alexidine Dihydrochloride Has Broad-Spectrum Activities against Diverse Fungal Pathogens

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ABSTRACT Invasive fungal infections due to *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans* constitute a substantial threat to hospitalized immunocompromised patients. Further, the presence of drug-recalcitrant biofilms on medical devices and emergence of drug-resistant fungi, such as *Candida auris*, introduce treatment challenges with current antifungal drugs. Worse, currently there is no approved drug capable of obviating preformed biofilms, which increase the chance of infection relapses. Here, we screened a small-molecule New Prestwick Chemical Library, consisting of 1,200 FDA-approved off-patent drugs against *C. albicans*, *C. auris*, and *A. fumigatus*, to identify those that inhibit growth of all three pathogens. Inhibitors were further prioritized for their potency against other fungal pathogens and their ability to kill preformed biofilms. Our studies identified the bis-biguanide alexidine dihydrochloride (AXD) as a drug with the highest antifungal and antibiofilm activity against a diverse range of fungal pathogens. Finally, AXD significantly potentiated the efficacy of fluconazole against biofilms, displayed low mammalian cell toxicity, and eradicated biofilms growing in mouse central venous catheters *in vivo*, highlighting its potential as a pan-antifungal drug.

IMPORTANCE The prevalence of fungal infections has seen a rise in the past decades due to advances in modern medicine leading to an expanding population of device-associated and immunocompromised patients. Furthermore, the spectrum of pathogenic fungi has changed, with the emergence of multidrug-resistant strains such as *C. auris*. High mortality related to fungal infections points to major limitations of current antifungal therapy and an unmet need for new antifungal drugs. We screened a library of repurposed FDA-approved inhibitors to identify compounds with activities against a diverse range of fungi in varied phases of growth. The assays identified alexidine dihydrochloride (AXD) to have pronounced antifungal activity, including against preformed biofilms, at concentrations lower than mammalian cell toxicity. AXD potentiated the activity of fluconazole and amphotericin B against *Candida* biofilms *in vitro* and prevented biofilm growth *in vivo*. Thus, AXD has the potential to be developed as a pan-antifungal, antibiofilm drug.

KEYWORDS *Candida albicans*, FDA, HTS, antifungal agents, biofilms, panfungal

Fungal pathogens responsible for invasive fungal infections (IFIs) are a leading cause of human mortality, killing approximately one and a half million people every year despite treatment with antifungal drugs (1). Of concern, the current incidence of fungal infection-related deaths is reported to be even higher than mortality due to tuberculosis or malaria (2). A vast majority of IFIs result from species belonging to *Cryptococcus*,

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Candida, or *Aspergillus* (3). However, fungi such as molds other than *Aspergillus*, and non-*albicans Candida* species, including the multidrug-resistant pathogen *Candida auris*, are becoming increasingly frequent and difficult to treat (4). Furthermore, other IFIs such as those due to Mucorales cause highly angioinvasive and tissue-destructive infections, which in many cases have mortality rates close to 100% (2).

The challenge in treatment of IFIs is directly linked to an ever-expanding population of immunocompromised patients requiring modern medical interventions and a paucity of currently approved antifungal agents (5, 6). Indwelling medical devices infected with fungi develop biofilms that are notoriously resistant to all classes of antifungal drugs and serve as a reservoir of infectious cells with direct access to the vasculature (7, 8). The current therapeutic armamentarium for IFIs is sparse, including only three classes of antifungal agents: polyenes, azoles, and echinocandins. These drugs have drawbacks, including significant limitations in spectrum of activity, human toxicity, and emergence of drug resistance, thereby underscoring a need for development of new antifungal agents (9, 10).

To fulfil this unmet need, we employed a high-throughput screening (HTS) assay to screen and characterize FDA (U.S. Food and Drug Administration)-approved, off-patent library drugs for their abilities to kill/inhibit three of the most invasive and drug-resistant human-pathogenic fungi, *Candida albicans*, *C. auris*, and *Aspergillus fumigatus*. This assay allowed us to identify core fungicidal molecules against all three pathogens. One of the leading compounds identified was a bis-biguanide dihydrochloride called alexidine dihydrochloride (AXD). AXD is an anticancer drug that targets a mitochondrial tyrosine phosphatase, PTPMT1, in mammalian cells and causes mitochondrial apoptosis (11). We found that AXD not only inhibited planktonic growth but also prevented biofilm formation as well as killing biofilms formed by a variety of drug-resistant and susceptible isolates of diverse fungal organisms. Further, when used in combination, AXD reduced the MIC of fluconazole and amphotericin B and rendered them efficacious against drug-resistant *C. albicans* biofilms. Finally, the antibiofilm property of AXD was also recapitulated in an *in vivo* mouse central venous catheter model of *C. albicans* biofilm formation. Overall, our studies warrant the further development of AXD as a panfungal antibiofilm drug, which could be used in combination therapeutics against diverse fungal pathogens.

RESULTS AND DISCUSSION

High-throughput screening (HTS) for identification of antifungal molecules. We used an HTS assay to test the ability of a commercially available, small-molecule library containing 1,233 FDA-approved compounds (New Prestwick Chemical [NPW] Library). We reckoned that repositioning existing off-patent drugs with known human safety and bioavailability profiles can accelerate the antifungal drug-discovery process without undergoing the arduous FDA approval process. These compounds were screened to identify a core set of inhibitors and fungicidals against *C. albicans*, *A. fumigatus*, and *C. auris*. The former two fungi represent two of the top four fungal pathogens causing IFIs with 40 to 70% mortality rates (3). *C. auris* is a newly emerging fungus that represents a serious global health threat due to its multidrug-resistant properties (12). We used cell viability as a parameter for prioritizing the broad-spectrum FDA-approved molecules as lead drugs for developing panfungal therapeutics. The purpose was to first identify a core set of molecules that could inhibit a diverse collection of fungi spanning different genera and species, under planktonic growth conditions.

HTS was performed in a 384-well plate screening format where the NPW library was screened against planktonic yeast or spore suspensions of the three fungal organisms, at a single concentration of 10 μ M. The spectrum of activity of these drugs was compared to clinically used azole drugs (fluconazole or voriconazole) at a concentration ranging from 0.03 to 32 μ g/ml. MICs of drugs were determined in agreement with the CLSI M27-A3 (for yeast) and M38-A2 (for filamentous fungi) reference standards for antifungal susceptibility testing (13, 14). After 3 days of incubation at 37°C, turbidity of the wells (OD₆₀₀) was measured and molecules displaying >50% reduction in turbidity

TABLE 1 Hits obtained from replicate primary screening of the New Prestwick Chemical Library against planktonic cells of *C. albicans*, *A. fumigatus*, and *C. auris*

Organism	Hits
<i>C. albicans</i>	Alexidine dihydrochloride, amphotericin B, antimycin A, butenafine hydrochloride, chloroxine, ciclopirox ethanolamine, clioquinol, clotrimazole, dequalinium dichloride, econazole nitrate, enilconazole, fluconazole, flucytosine, haloprogin, isoconazole, isoxsuprine hydrochloride, itraconazole, ketoconazole, methyl benzethonium chloride, pyrvinium pamoate, sertaconazole nitrate, sulconazole nitrate, terconazole, thimerosal, thonzonium bromide, tioconazole, voriconazole
<i>A. fumigatus</i>	Alexidine dihydrochloride, butenafine hydrochloride, isoxsuprine hydrochloride, clioquinol, thimerosal, dequalinium dichloride, pyrvinium pamoate, haloprogin, chlorhexidine, voriconazole, amphotericin B, antimycin A, econazole nitrate, enilconazole, methyl benzethonium chloride, tioconazole
<i>C. auris</i>	Alexidine dihydrochloride, butenafine hydrochloride, chloroxine, clioquinol, thimerosal, haloprogin

compared to control non-drug-treated wells (MIC_{50}) were considered primary “hits.” Z' factor was calculated as a parameter of HTS quality, and an average Z' factor of 0.75 was computed for our assays (a value of >0.5 represents an excellent quality of HTS) (15).

From this hit list, a core set of molecules that inhibited planktonic growth of all three fungi as identified by $>50\%$ growth inhibition measured by MIC were identified and shortlisted. *C. albicans* was sensitive to fluconazole at concentrations of <0.125 to $0.25 \mu\text{g/ml}$, as has been reported previously (16), while, consistent with its drug-resistant nature, *C. auris* was resistant to fluconazole with MICs of $>16 \mu\text{g/ml}$ (see Table 2). *A. fumigatus* succumbed to voriconazole at $0.25 \mu\text{g/ml}$, similarly to previously reported antifungal drug susceptibility studies (16). Recently, Siles et al. investigated the ability of Prestwick Chemical Library to specifically inhibit *C. albicans* biofilms and revealed 38 pharmacologically active agents against the fungus (17). While our study also identified a number of molecules individually inhibiting the three fungi, respectively (Table 1), only the following six compounds were successful at inhibiting all three organisms: chloroxine, thimerosal, alexidine dihydrochloride, haloprogin, clioquinol, and butenafine hydrochloride (Table 1). NPW contains a number of antifungal drugs, such as imidazoles, triazoles, and the polyene class of drugs. *C. auris* was by far the most resistant fungus, inert against the azoles and polyenes in the library. The six molecules were further evaluated for their ability to curtail biofilm formation as well as kill preformed biofilms developed by the three fungi. Table 1 lists primary hits that were confirmed and narrowed after a repeat screen under similar planktonic conditions.

Secondary assays for determination of antibiofilm activity. Wells with *C. albicans*, *C. auris*, and *A. fumigatus* were either treated with inhibitors at the time of yeast/spore inoculation (start of biofilm initiation) or allowed to grow without drugs for 48 h to allow biofilm development (mature biofilm). This assay was performed in a 96-well microtiter plate assay, as previously reported by us (18). For the effect on formation of biofilm, all six inhibitors could inhibit biofilm formation in the three fungal organisms, as adjudged by a significant decrease in turbidity of the media in the wells 48 h following incubation with the drugs (data not shown). However, only two drugs, alexidine dihydrochloride and thimerosal, could significantly kill 80% of mature biofilm community at the tested concentration of $<10 \mu\text{M}$ (Fig. 1A; see also Fig. S1A in the supplemental material). We chose to focus our attention to studying alexidine dihydrochloride (AXD), since it was more attractive with respect to drug development, having indications for use as an antibacterial and antiplaque agent and with limited side effects (19–21).

Dose-response assays of AXD. AXD was first evaluated in planktonic and biofilm dose-response assays and further tested for its ability to inhibit growth of other fungal pathogens, including drug-resistant clinical isolates, a number of non-*albicans* *Candida* spp., and members of the Mucorales family. The results for this study are described in Table 2, which lists the fungal strains used for evaluation of the efficacy of AXD, under three different growth conditions—planktonic, biofilm inhibition, and preformed biofilms, compared to the MIC of the control azole antifungal drugs. For example, AXD displayed activity against most *Candida* spp.; MIC values of $\leq 1.5 \mu\text{g/ml}$ were observed

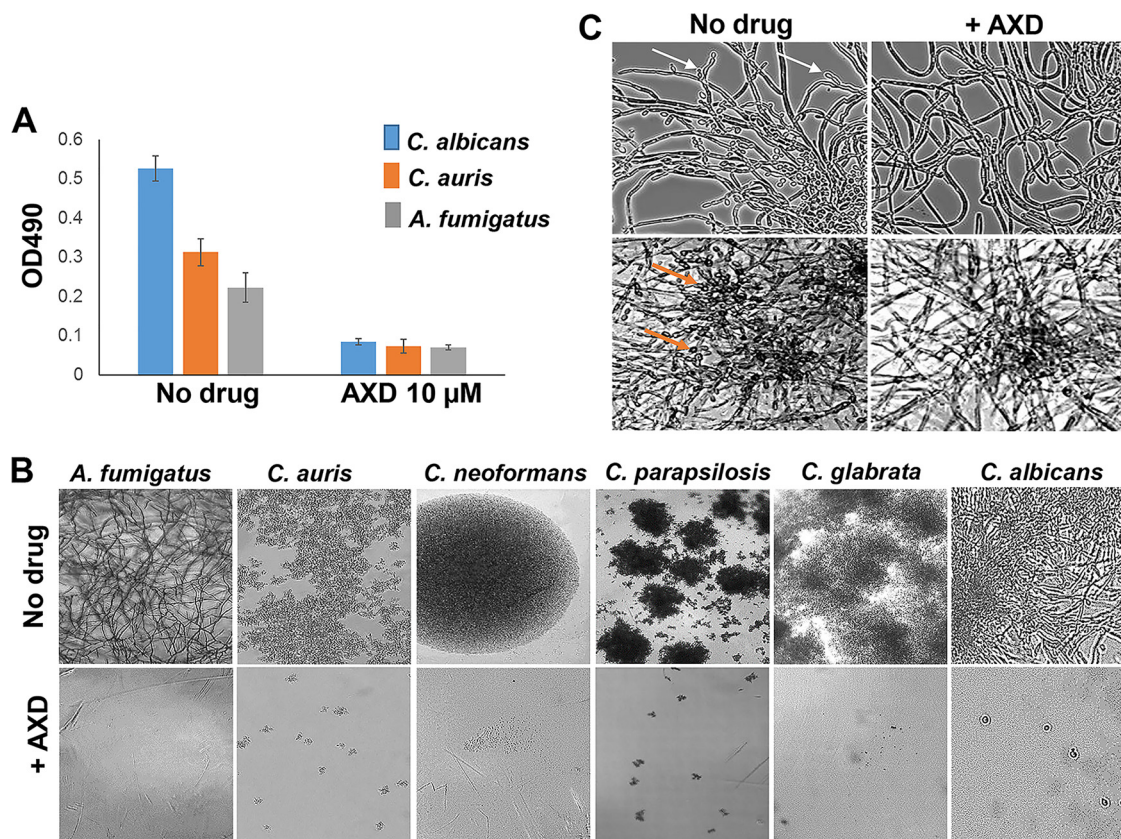


FIG 1 Inhibition of biofilm growth, *C. albicans* biofilm dispersal, and abrogation of planktonic growth in diverse fungi by alexidine dihydrochloride (AXD). (A) Fungal cells were allowed to form a biofilm for 48 h and treated for 24 h with 10 μ M AXD. Biofilm inhibition was as determined by XTT reading (OD₄₉₀). (B) Fungal yeast cells or spores were incubated with different concentrations of AXD under planktonic conditions. Inhibition of growth and filamentation of the fungi visualized by phase-contrast microscopy (20 \times magnification), at their respective AXD MIC₈₀s. (C) *C. albicans* planktonic hyphae (top two panels) and biofilms (bottom two panels) were treated for 12 h with 150 ng/ml of AXD. AXD inhibited lateral yeast production from hyphal cells and hyphal layers of biofilms, as visualized microscopically. Arrows point to lateral yeasts.

for all isolates tested under planktonic conditions, with the exception of *Candida parapsilosis* and *Candida krusei*. Interestingly, AXD also displayed striking activity against clinically relevant fluconazole-resistant *Candida* isolates: *C. albicans* (CA2, CA6, and CA10), *C. glabrata* (CG2 and CG5), *C. parapsilosis* (CP5), and *C. auris* (CAU-09 and CAU-03). Furthermore, the MIC values of AXD against *C. neoformans* were comparable to the MIC values for fluconazole.

In the case of filamentous fungi, low AXD MIC₅₀ values of 1.5 to 3 μ g/ml were observed for all filamentous fungi (Mucorales and *Aspergillus* spp., plates read at 48 h), including the molds *L. corymbifera* and *S. apiospermum* (read at 72 h), which have poor outcomes with current clinically available antifungal drugs (22). Inhibition of planktonic growth by AXD monitored microscopically revealed a complete inhibition of filamentation or proliferation of the imaged fungi (Fig. 1B). Of particular importance was the finding that AXD was able to decimate at low concentrations (1.5 to 6 μ g/ml) mature biofilms of *Candida*, *Cryptococcus*, and *Aspergillus* spp. that are known to be resistant to almost all classes of antifungal drugs (Table 2; also see Fig. S2 for AXD activity on *Candida* spp.). In fact, at 10-fold-lower concentrations (150 ng/ml) of planktonic MICs, AXD could inhibit lateral yeast formation and biofilm dispersal in *C. albicans* (Fig. 1C). The dispersal of lateral yeast cells from a biofilm biomass is the link between contaminated catheters and disseminated candidiasis (8, 23). Inhibition of dispersal with just nanomolar levels of AXD can help seal the biofilm reservoir and curtail further proliferation and robustness of a biofilm.

TABLE 2 MICs of AXD against clinical isolates of different fungal species versus fluconazole or voriconazole^a

Isolate	MIC ($\mu\text{g/ml}$)						
	Planktonic azole	Planktonic AXD		Biofilm-inhibitory AXD		Mature biofilm AXD	
		50%	80%	50%	80%	50%	80%
	Fluconazole						
CA:SC5314	<0.5	0.79	0.73	0.73	0.73	3	12
CA1	<0.125	1.5	1.5	0.73	0.73	6	12
*CA2	16	0.94	0.94	1.5	1.5	3	20
CA4	<0.125	1.5	1.5	0.73	0.73	3	12
*CA6	16	1.5	1.5	1.5	1.5	1.5	6
*CA10	32	1.5	1.5	0.73	1.5	3	6
CG1	2	0.73	1.5	0.73	1.5	3	6
CG2	32	0.73	1.5	0.15	1.5	3	6
CG3	2	0.73	1.5	0.73	1.5	3	6
CG4	4	1.10	1.1	1.5	3	3	6
*CG5	256	1.14	1.14	1.5	3	3	12
CP1	0.25	1.5	3	3	3	3	3
CP2	0.25	3	6	3	3	3	6
CP3	2	3	6	3	3	6	>12
CP4	≤ 0.12	1.4	1.4	2.5	3	3	3
*CP5	64	2.29	3	4	6	6	6
CK	NA	2.21	3	2.5	6	6	6
CT1	2	0.84	0.84	0.84	1.5	1.5	3
*CT2	>256	0.84	0.84	0.84	1.5	1.5	3
CN1	1	0.73	0.73	1.5	6	3	6
CN2	0.5	0.73	0.73	0.73	3	3	3
CN3	1	1.5	1.5	1.5	3	1.5	1.5
CAU-03	32	0.73	1.5	3	3	3	3
CAU-09	16	1.5	1.5	6	6	3	6
	Posaconazole						
<i>R. delemar</i> 99.880	0.25	1.5	1.5	NT	NT	NT	NT
<i>R. oryzae</i> 99.892	0.25	1.5	3	NT	NT	NT	NT
	Voriconazole						
<i>M. circinelloides</i> 131	8	0.73	3	NT	NT	NT	NT
<i>L. corymbifera</i> 008049	>32	3	6	NT	NT	NT	NT
<i>C. bertholletiae</i> 182	8	3	6	NT	NT	NT	NT
<i>S. apiospermum</i> DI16-478	8	1.5	1.5	NT	NT	NT	NT
AF293	0.25	0.73	3	0.73	3	6	6
AF1	1	0.73	3	0.73	3	6	6
AF2	0.25	1.5	6	1.5	3	6	6
AF3	0.25	1.5	3	1.5	3	6	6

^aCA, *C. albicans*; CAU, *C. auris*; CG, *C. glabrata*; CP, *C. parapsilosis*; CK, *C. krusei*; CN, *C. neoformans*; AF, *A. fumigatus*; NA, not available; NT, not tested. The asterisks signify drug-resistant clinical isolates.

Alexidine dihydrochloride is a bis-biguanide in which the common 2-ethylhexyl chain has been attached to each biguanide unit and the two units are linked by a 1,6-hexanediamine chain (Fig. S3A). This compound, initially identified for its antibacterial properties, is also found as an inducer of mitochondrial dysfunction and apoptosis (11, 24). AXD has been proposed in several studies as an antiplaque agent and mouthwash and with potential to be used in endodontic treatment to eliminate biofilms (19, 20, 25). These reports, along with our present findings, serve as precedents for the development of AXD as an antibiofilm agent. Another bis-biguanide with structural similarity to AXD, metformin, has recently been shown to have antifungal activity (and synergistic potentiation of clinically used antifungal drugs) against *C. glabrata*, albeit only at physiologically inapt high concentrations (26). AXD, on the other hand, is active even at levels as low as 0.75 $\mu\text{g/ml}$ against an array of fungal species in our current study. In fact, AXD has been reported to have activity against the fungus *C. neoformans*, by targeting phospholipases (27). Whether specific inhibition of fungal phospholipases is the cause of AXD's antifungal activity against a spectrum of pathogenic fungi is unknown and remains to be explored in future studies.

Mammalian cell cytotoxicity assays and synergy of AXD with fluconazole. Considering that AXD displayed enhanced efficacy against fungal organisms, we eval-

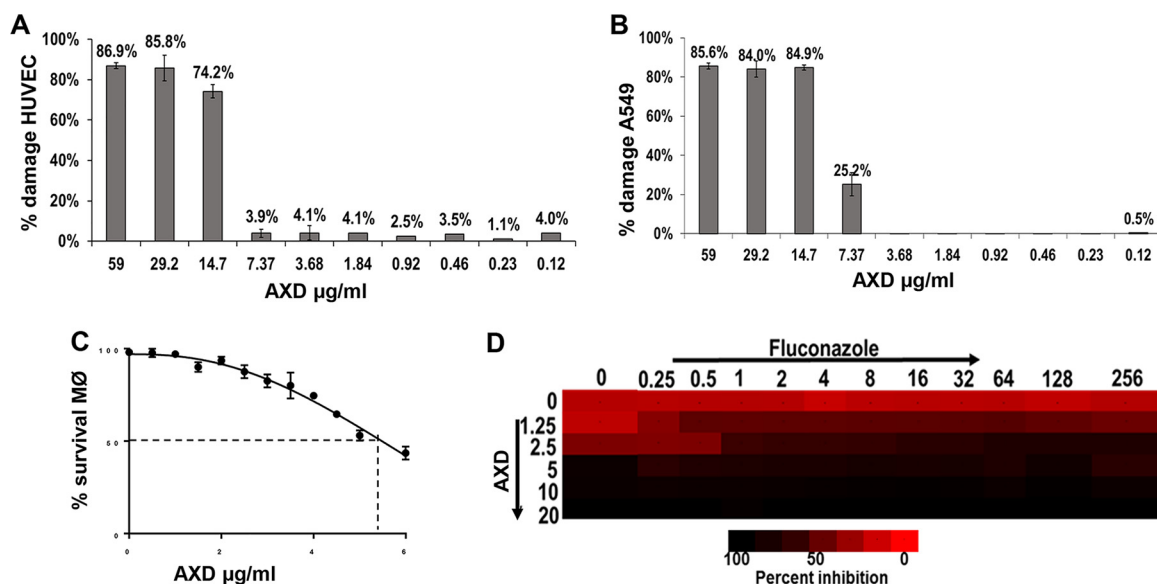


FIG 2 Toxicity of AXD on host cells and on biofilm killing in combination with fluconazole. Different concentrations of AXD were incubated with HUVECs (A), lung A549 cells (B), or macrophages (C) for 24 h at 37°C, for testing the CC_{50} of the drug to the respective cell lines. (D) *C. albicans* biofilms were developed for 48 h and then treated with different concentrations of AXD and fluconazole in a checkerboard format. Metabolic activity of biofilm cells was measured by the XTT assay. Bright red represents growth above the MIC_{50} , dull red represents growth at the MIC_{50} , and black/dark red represents growth below the MIC_{50} .

uated the extent of its cell toxicity (CC_{50}) to mammalian cells. The results showed that AXD resulted in 50% killing of HUVECs and lung epithelial cells, at concentrations 5- to 10-fold higher than the MIC required to kill planktonically growing fungal pathogens (CC_{50} of $>7.37 \mu\text{g/ml}$ versus planktonic MIC_{50} of 0.73 to 1.5 $\mu\text{g/ml}$) (Fig. 2A and B). Previous studies have reported similar cytotoxicity levels of AXD against various other cell lines (19, 27, 28).

We further tested the toxicity of AXD for a human bone marrow-derived macrophage cell line to understand its effect on the immune cells. AXD displayed a slightly higher toxicity to the macrophages than the mammalian tissue cell lines, with a CC_{50} of over 5 $\mu\text{g/ml}$ (Fig. 2C). A similar study was also done to test the impact of AXD toxicity on HL60 monocyte proliferation. HL60 cells stained with CFSE were treated with various concentrations of AXD, or the control PMA (phorbol 12-myristate 13-acetate) as a positive stimulant that induces cellular proliferation. Inhibition of cellular proliferation corresponds to toxicity, and the concentration of AXD that could prevent early cell division in HL60 cells was examined. As expected, cells stimulated by PMA showed proliferation, while those treated with the highest dose of AXD (10 $\mu\text{g/ml}$) did not divide. AXD at 5 $\mu\text{g/ml}$ prevented cell division in the human bone marrow-derived HL60 cells (Fig. S3B). This level of toxicity matched the macrophage CC_{50} value. We note that concentrations detrimental to host cells are at least 3- to 4-fold higher than AXD levels required to inhibit planktonic cells of many different fungi, including *C. albicans*. These moderately low cytotoxicities of the FDA-approved drug pave the way to a potential repurposing of AXD as an antifungal agent and warrant its further development into a compound with higher efficacy and bioavailability and less toxicity.

This inhibitory potential was further highlighted in our studies evaluating synergistic action of AXD in combination with fluconazole against mature *C. albicans* biofilms. Fluconazole is completely inert against *C. albicans* biofilms, with an MIC_{50} of $>256 \mu\text{g/ml}$ (this study and references 7, 29, and 30). When used together, AXD at 1.25 $\mu\text{g/ml}$ strikingly reduced the MIC_{50} of fluconazole from $>256 \mu\text{g/ml}$ to a clinically relevant 1 $\mu\text{g/ml}$ (Fig. 2D), providing an FIC index of 0.42, which indicated a synergistic interaction (30). These results further emphasize AXD's prospects as an antibiofilm agent, especially due to its ability to lower MICs of fluconazole, highlighting the possibility of bringing a biofilm-redundant drug back into clinical use.

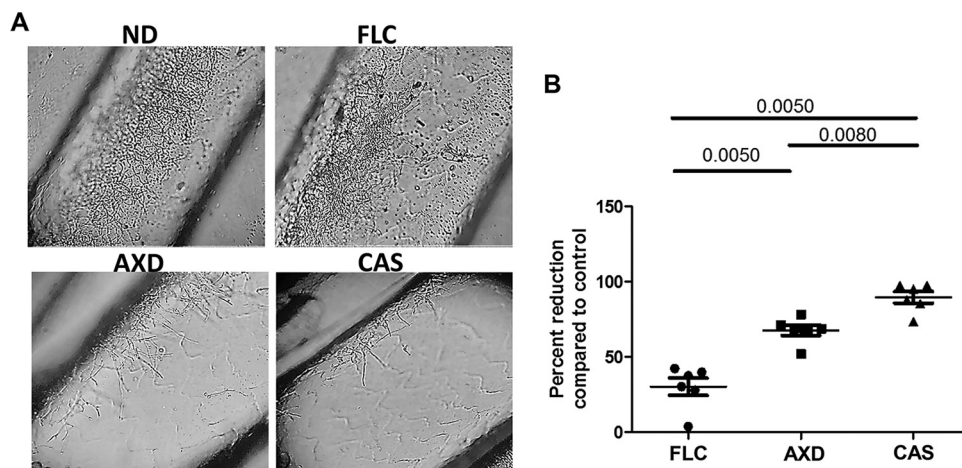


FIG 3 Impact of AXD, fluconazole (FLC), and caspofungin (CAS) as lock therapy against *C. albicans* biofilm cells in an *in vivo* catheter model. (A) Biofilms were grown for 24 h followed by intraluminal drug treatment for 24 h. Following compound exposure, the catheters were removed for microscopy and CFU enumeration. Each of the four panels represent a 40 \times magnification under phase-contrast microscope. Panel columns: no drug treatment (ND), control biofilm treated with saline; FLC, 125- μ g/ml fluconazole exposure; AXD, catheters exposed to AXD at 3 μ g/ml; CAS, catheters exposed to 0.25 μ g/ml caspofungin. (B) After ND or drug treatment, catheters were cut into pieces, vortexed, and sonicated to release adhered cells in sterile PBS, and dilutions of the suspension were plated on solid medium for CFU enumeration. Results are presented as percent biofilm reduction in drug-treated catheters compared to the untreated catheter biofilms and analyzed statistically by using a nonparametric *t* test. A *P* value of <0.05 is significant.

Inhibition of biofilm *in vivo* by AXD. Our studies showed that AXD could arrest growth and kill biofilm cells formed by various *Candida* species, *C. neoformans*, and *A. fumigatus* in *in vitro* assays. We next examined the ability of AXD to decimate pre-formed biofilms in an *in vivo* model. For this study, we chose to focus on biofilm formation by *C. albicans*, since a murine biofilm model has been well established in this fungus and used for testing the effects of established and new antifungal agents (31). The effect of the drugs on the 24-h-old biofilms growing in the jugular vein catheters of mice was visualized microscopically, which revealed significantly lower density of the biofilms in catheters treated with AXD and caspofungin, versus the control untreated catheters (Fig. 3A). In fact, fungal CFU determination revealed that AXD inhibited 67% of fungal biofilm growth and viability, compared to the control untreated biofilms (Fig. 3B). As expected, caspofungin (an antifungal drug known to be hyperactive against *C. albicans* biofilms) decimated >90% of the biofilm community growing within the catheters. On the other hand, fluconazole (a drug with enhanced activity against planktonic fungi, but with limited activity against biofilm cells) was found to reduce biofilms by only 30% (*P* value = 0.028) (Fig. 3B). Overall, our data show that AXD can inhibit biofilm growth *in vivo*. A better understanding of the pharmacokinetics/pharmacodynamics of AXD could be invaluable in assessing its utility as a systemic antifungal drug, especially in a disseminated mouse model of fungemia.

In summary, our HTS identified alexidine dihydrochloride to have profound activity against various growth forms of fungi: planktonic, biofilm, and biofilm dispersal. AXD was fungicidal to a number of different pathogenic fungi, including common as well as emerging drug-resistant pathogens. The fact that AXD retains its activity against azole-resistant clinical isolates indicates its potential use in recalcitrant fungal infections. Importantly, AXD reduces the MIC of fluconazole, a clinically used first-line antifungal drug, ironically considered dispensable for biofilm treatment, thereby pointing to its extended utility as an antibiofilm combination drug. Perhaps the most intriguing activity of AXD was seen against Mucorales, including *Rhizopus*, a species that leads to devastating infections and very poor outcomes in patients, despite conventional antifungal treatment. Furthermore, the drug was also potent against those fungi that are therapeutically unmanageable in clinics with current antifungal

agents such as *L. corymbifera* and *S. apiospermum*. Future studies will focus on the mechanism of action of AXD at a molecular level and evaluate its feasibility as a pan-antifungal drug to combat infections in different clinical settings.

MATERIALS AND METHODS

Strains, media, and culture conditions. The following fungal strains were used in this study: *C. albicans* strain SC5314, which is a human clinical isolate recovered from a patient with generalized candidiasis (32), and several clinical isolates of *Candida* spp. received from the Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio—fluconazole-sensitive *C. albicans* CA1 and CA4; fluconazole-resistant *C. albicans* CA6 and CA10; *C. glabrata* fluconazole-sensitive CG1 and CG3 and fluconazole-resistant CG2; *C. parapsilosis* CP1, CP2, and CP3; *C. neoformans* CN1, CN2, and CN3; and *A. fumigatus* AF1, AF2, and AF3. Some *Candida* strains were also obtained from the Division of Infectious Disease, Massachusetts General Hospital, Boston, MA: fluconazole-resistant strains of *C. albicans* CA2, *C. parapsilosis* CP4 and CP5, *C. krusei* CK, and *C. tropicalis* CT2. The two *C. auris* isolates CAU-03 and CAU-09 were a kind gift from Shawn Lockhart, Centers for Disease Control and Prevention (CDC), and the filamentous fungi, including *Rhizopus delemar* 99.880 and *Rhizopus oryzae* 99.892, *L. corymbifera* 008049, *C. bertholletiae* 182, *M. circinelloides* 131, and *S. apiospermum* D116-478, were a part of the fungal bank at Division of Infectious Diseases, Los Angeles Biomedical Research Center. All cultures were maintained by subculture on yeast-peptone-dextrose medium (YPD) at 37°C, and stocks of these cultures were stored in 20% glycerol at –80°C.

HTS. Screening was performed at the Molecular Screening Shared Resource facility at the University of California, Los Angeles. A total of 50 μ l of 1×10^4 fungal yeast cells/ml (*C. albicans* and *C. auris*) or spores (*Aspergillus*) was suspended in RPMI 1640 supplemented with L-glutamine (Cellgro), buffered with 165 mM morpholinepropanesulfonic acid (MOPS), and plated into individual 384-well plates using an automated Multidrop 384 system (Thermo Labsystems). The New Prestwick Chemical Library consisting of 1,233 drugs was used to pin one compound per well at 10 μ M final concentration, using a Biomek FX liquid handler. Forty-eight hours later the plates were scanned with a Flex Station II 384-well plate reader (Molecular Devices) to measure turbidity (OD₆₀₀) of the wells. Molecules displaying >80% reduction in turbidity compared to control non-drug-treated wells (MIC₈₀) were considered primary “hits.” Compounds commonly inhibiting all three fungal organisms were prioritized for planktonic dose-response assays and for their activity against biofilm growth.

Dose-response assays. Dose-response assay of AXD against planktonically grown fungi was performed in agreement with the CLSI M27-A3 (for yeast) and M38-A2 (for filamentous fungi) reference standards for antifungal susceptibility testing (13, 14). Each drug was used in the concentration range of 0.19 μ g/ml to 24 μ g/ml, and the MIC of AXD was compared to the MIC of fluconazole, posaconazole, or voriconazole, as controls. All strains described in Table 2 were tested at the LA Biomedical Research Institute; however, several of the *Candida* strains were also verified for their susceptibility to AXD independently at Massachusetts General Hospital. Inhibition of planktonic growth or filamentation due to drug treatment was also visualized and imaged using bright-field microscopy. Microscopy was also used to directly visualize lateral yeast formation from planktonic *C. albicans* hyphae or lateral yeast cells formed on the surface of the biofilms (dispersal) using microtiter plates.

Biofilm growth and drug susceptibility testing. Biofilms of *Candida* spp., *C. neoformans*, and *A. fumigatus* were developed in 96-well microtiter plates, and susceptibility of the biofilm cells to AXD or thimerosal was determined as described previously (33, 34). Biofilms were initiated in either the presence or absence of the drugs, or the drugs were tested on 48-h preformed biofilms, for efficacy evaluation. Inhibition of biofilm growth was measured by a standard colorimetric assay (XTT) that measures metabolic activity of the biofilm cells (18). Absorbance at 490 nm was measured using an automated plate reader. Biofilms formed by several other *Candida* spp. were further studied for their susceptibility to AXD.

Potential of AXD for synergistic use with fluconazole against *C. albicans* biofilms was investigated using a checkerboard assay, where dilutions of fluconazole (0.25 to 250 μ g/ml) and AXD (0.3 to 2 μ g/ml) were examined alone and in combination. Biofilm killing was measured by XTT assay. Drug concentration associated with 50% reduction in optical density compared to the no-drug control wells (EC₅₀) was determined. The fractional inhibitory concentration (FIC) was then calculated as follows: [(EC₅₀ of drug A in combination)/(EC₅₀ of drug A alone)] + [(EC₅₀ of drug B in combination)/(EC₅₀ of drug B alone)]. Values of ≤ 0.5 revealed synergy, those of > 0.5 but < 2 indicated no interaction, and those of > 2 were antagonistic (30).

Mammalian cell toxicity assays. Primary human umbilical vascular endothelial cells (HUVECs) and human lung carcinoma-derived A549 epithelial cell lines were used to determine the cytotoxicity of AXD. HUVECs were isolated and propagated by the method of Jaffe et al. (35). The cells were grown in M-199 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 10% defined bovine calf serum, and 2 nM L-glutamine, with penicillin and streptomycin. Second- or third-passage endothelial cells were grown on collagen matrix on 96-well microtiter plates. Treatment with AXD was conducted in M-199 medium.

A549 cells were purchased from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. A549 cells (1.5×10^5 /well) were used to seed 96-well plates and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h. The medium was then removed by aspiration, and the cells were washed twice with phosphate-buffered saline. Treatment with AXD was conducted with DMEM supplemented with 1% FBS.

Different concentrations of AXD in respective media were introduced into the cell lines and incubated for 24 h at 37°C in 5% CO₂. The extent of cellular damage to both cell lines caused by AXD was quantified by a chromium release assay (36). Briefly, confluent mammalian cells were incubated overnight in respective media containing Na₂ ⁵¹CrO₄ (6 μCi per well; ICN Biomedicals, Irvine, CA). The next day, the unincorporated tracer was aspirated and the wells were rinsed three times with warm HBSS. Two hundred microliters of media containing various concentrations of AXD (ranging from 0.12 to 59 μg/ml) was added to each well, and the plate was incubated for 24 h at 37°C in 5% CO₂. At the end of the incubation, 100 μl of medium was gently aspirated from each well, after which the cells were lysed by the addition of 6 N NaOH. The lysed cells were aspirated, and the wells were rinsed twice with RadicWash (Atomic Products, Inc., Shirley, NY). These rinses were added to the lysed cells, and the ⁵¹Cr activity of the medium and the cell lysates was determined. Control wells containing no drug were processed in parallel to measure the spontaneous release of ⁵¹Cr. After corrections were made for the differences in the incorporation of ⁵¹Cr in each well, the specific release of ⁵¹Cr was calculated by the following formula: (2 × experimental release – 2 × spontaneous release)/(total incorporation – 2 × spontaneous release).

Cytotoxicity to immune cells. Wild-type C57BL/6 primary bone marrow-derived macrophages were cultured by plating bone marrow cells in 50 ng/ml of M-CSF (Peprotech, Rocky Hill, NJ) in complete RPMI (RPMI 1640 with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, and 1% penicillin-streptomycin) for 7 days and then counted and seeded at 1 × 10⁵ in 100 μl of complete RPMI overnight to allow for adhesion.

To examine cytotoxicity of AXD, bone marrow-derived macrophages were incubated in various concentrations of AXD for 24 h and stained with DAPI (Invitrogen, Carlsbad, CA) for viability assessment using an inverted epifluorescence microscope (Olympus IX70, Center Valley, PA) using 10× objective, with an X-cite 120 metal halide light source (EXFO, Mississauga, ON, Canada). Percent cell viability was determined using 1 – (DAPI-positive cells divided by total cells by phase contrast) × 100.

AXD was also examined for its capacity to block proliferation of a human promyelocytic cell line, HL-60. Cells were stained with 2 mM CFSE (carboxyfluorescein succinimidyl ester) for 5 min and washed with 1 × RPMI medium three times. This dye is commonly used to measure cell proliferation; with each cell division the amount of CFSE is diluted in half, which can be observed via flow cytometry (37). After the staining, the cells were counted and adjusted at a cell density of 5 × 10⁶ cells/ml and plated at 100 μl/well in a round-bottom 96-well plate. Twofold serially diluted AXD was added in wells containing cells. The final drug concentration obtained was between 0.004 and 10 μg/ml. Non-drug-treated and unstained cells in a number of wells were included as controls. Plates were incubated at 37°C for 48 h to allow the cell proliferation. After 48 h, the cells were collected and acquired in a flow cytometer. The unstained cells were used to gate the CFSE-positive HL60 cells. The shift in the peak of CFSE⁺ HL60 cells was considered proliferating cells.

In vivo biofilm drug susceptibility. A mouse central venous catheter infection model was used for biofilm studies as previously described (31). These *in vivo* experiments were approved by the Los Angeles Biomedical Research Institute, Harbor-UCLA IACUC. Briefly, we used catheterized 8-week-old C57BL/6 male mice, purchased from Charles River Labs (Wilmington, MA), where the surgery was performed. The surgery involves insertion of a Silastic catheter into the jugular vein of the mice. Patency is tested, and the catheter is filled with heparin lock solution and plug-sealed. Following receipt of the jugular vein-catheterized mice, the catheters were instilled with 25 μl of *C. albicans* inoculum of 5 × 10⁶ cells/ml (entire catheter volume) using a 23-gauge blunt-ended needle after removal of the plug and the lock solution (the plug was put back in place after inoculation). Cells were allowed to develop biofilms for 24 h, after which the catheters were treated with 3 μg/ml AXD for 48 h. Biofilms growing in replicate mouse catheters were also subjected to fluconazole (250 μg/ml) or caspofungin (0.125 μg/ml) treatment, as comparative controls. The catheters were cut laterally and imaged under a phase-contrast microscope to visualize the morphology of the cells growing within the catheters of the individual groups. Additionally, the distal 2 cm of the catheters was cut into small pieces, vortexed vigorously, and homogenized for plating on YPD plates for viability count measurements.

Statistical methods. All *in vitro* secondary assays were done in triplicate and repeated once. Experiments were conducted in a randomized fashion and subjected to unpaired two-tailed *t* tests and/or ANOVA with Kruskal-Wallis posttest to determine significance of results (for *P* ≤ 0.05). For *in vivo* studies, differences in catheter fungal burden between the four groups (6 mice per group) were presented as percent reduction in CFU in the individual drug-treated groups compared to the control untreated mice. A two-tailed *t* test with a *P* value of <0.05 was considered significant.

Data availability. Compounds identified after the first primary screening of the NPW library against planktonic cells of *C. albicans*, *A. fumigatus*, and *C. auris* are presented in Table S1 in the supplemental material. Any further data are available upon request from the corresponding author.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mSphere.00539-18>.

FIG S1, TIF file, 2.9 MB.

FIG S2, TIF file, 2.7 MB.

FIG S3, TIF file, 2.9 MB.

TABLE S1, XLSX file, 0.01 MB.

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