

1 **Alexidine dihydrochloride has broad spectrum activities against diverse fungal**
2 **pathogens.**

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6 **Running title:** Alexidine dihydrochloride has pan-antifungal activity

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28 Abstract word count: 175

29 Manuscript word count: 4267

30 **Abstract.**

31 Invasive fungal infections due to *Candida albicans*, *Aspergillus fumigatus* and
32 *Cryptococcus neoformans*, constitute a substantial threat to hospitalized,
33 immunocompromised patients. Further, the presence of drug-recalcitrant biofilms on
34 medical devices, and emergence of drug-resistant fungi such as *Candida auris*,
35 introduce treatment challenges with current antifungal drugs. Worse, currently there is
36 no approved drug capable of obviating preformed biofilms which increases the chance
37 of infection relapses. Here, we screened a small molecule Prestwick Chemical Library,
38 consisting of 1200 FDA approved off-patent drugs, against *C. albicans*, *C. auris* and *A.*
39 *fumigatus*, to identify those that inhibit growth of all three pathogens. Inhibitors were
40 further prioritized for their potency against other fungal pathogens, and their ability to kill
41 preformed biofilms. Our studies identified the bis-biguanide Alexidine dihydrochloride
42 (AXD), as a drug with the highest antifungal and anti-biofilm activity against a diverse
43 range of fungal pathogens. Finally, AXD significantly potentiated the efficacy of
44 fluconazole against biofilms, displayed low mammalian cell toxicity, and eradicated
45 biofilms growing in mice central venous catheters *in vivo*, highlighting its potential as a
46 pan-antifungal drug.

47 **Importance.**

48 The prevalence of fungal infections has seen a rise in the past decades due to
49 advances in modern medicine leading to an expanding population of device-associated
50 and immunocompromised patients. Furthermore, the spectrum of pathogenic fungi has
51 changed, with the emergence of multi-drug resistant strains such as *C. auris*. High
52 mortality related to fungal infections point to major limitations of current antifungal

53 therapy, and an unmet need for new antifungal drugs. We screened a library of
54 repurposed FDA approved inhibitors to identify compounds with activities against a
55 diverse range of fungi, in varied phases of growth. The assays identified Alexidine
56 dihydrochloride (AXD) to have pronounced antifungal activity including against
57 preformed biofilms, at concentrations lower than mammalian cell toxicity. AXD
58 potentiated the activity of fluconazole and amphotericin B against *Candida* biofilms *in*
59 *vitro*, and prevented biofilm growth *in vivo*. Thus AXD has the potential to be developed
60 as a pan-antifungal, anti-biofilm drug.

61

62 **Introduction.**

63 Fungal pathogens responsible for invasive fungal infections (IFIs) are a leading cause of
64 human mortality, killing approximately one and a half million people every year, despite
65 treatment with antifungal drugs (1). Of concern, the current incidence of fungal-related
66 deaths is reported to be even higher than mortality due to tuberculosis or malaria (2). A
67 vast majority of IFIs result from species belonging to *Cryptococcus*, *Candida* or
68 *Aspergillus* (3). However, fungi such as molds other than *Aspergillus*, and non-*albicans*
69 *Candida* species including the multi-drug resistant pathogen *C. auris*, are becoming
70 increasingly frequent and difficult to treat (4). Furthermore, other IFIs such as those due
71 to Mucorales cause highly angioinvasive and tissue-destructive infections which in
72 many cases have mortality rates close to 100% (2).

73 The challenge in treatment of IFIs is directly linked to an ever expanding population of
74 immunocompromised patients requiring modern medical interventions, and a paucity of

75 currently approved antifungal agents (5, 6). Indwelling medical devices infected with
76 fungi, develop biofilms that are notoriously resistant to all classes of antifungal drugs,
77 and serve as a reservoir of infectious cells with direct access to the vasculature (7, 8).
78 Current therapeutic armamentarium for IFIs is sparse, including only three classes of
79 antifungal agents: polyenes, azoles, and echinocandins. These drugs have drawbacks
80 including significant limitations in spectrum of activity, human toxicity and emergence of
81 drug resistance, thereby underscoring a need for development of new antifungal agents
82 (9, 10).

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

98 as a pan-fungal anti-biofilm drug, which could be used in combination therapeutics
99 against diverse fungal pathogens.

100

101 **Results and discussion**

102 **High throughput screening (HTS) for identification of antifungal molecules.**

103 We used a HTS assay to test the ability of a commercially available, small molecule
104 library containing 1233 FDA approved compounds (New Prestwick Chemical [NPW]
105 Library). We reckoned that repositioning existing off-patent drugs with known human
106 safety and bioavailability profiles, can accelerate the antifungal drug-discovery process
107 without undergoing the arduous FDA approval process. These compounds were
108 screened to identify a core set of inhibitors and fungicides against *C. albicans*, *A.*
109 *fumigatus* and *C. auris*. The former two fungi represent two of the top four fungal
110 pathogens causing IFIs with 40-70% mortality rates (3). *C. auris* is a newly emerging
111 fungus that represents a serious global health threat due to its multi-drug resistant
112 properties (12). We used cell viability as a parameter for prioritizing the broad spectrum
113 FDA-approved molecules as lead drugs for developing pan-fungal therapeutics. The
114 purpose was to first identify a core set of molecules that could inhibit a diverse collection
115 of fungi spanning different genus and species, under planktonic growth conditions.

116 HTS was performed in a 384-well plate screening format, where the NPW library was
117 screened against planktonic yeast or spore suspensions of the three fungal organisms,
118 at a single concentration of 10 μ M. The spectrum of activity of these drugs was
119 compared to clinically used azole drugs (fluconazole or voriconazole) at a concentration

120 ranging from 0.03 to 32 μ g/ml. MIC of drugs were determined in agreement with the
121 CLSI M27-A3 (for yeast) and M38-A2 (for filamentous fungi) reference standards for
122 antifungal susceptibility testing (13, 14). After three days of incubation at 37°C, turbidity
123 of the wells (OD600) was measured and molecules displaying >50% reduction in
124 turbidity compared to control non-drug treated wells (MIC50) were considered as
125 primary “hits”. Z' factor, was calculated as a parameter of HTS screening quality, and
126 an average Z' factor of 0.75 was computed for our assays (a value of >0.5 represents
127 an excellent quality of HTS)(15).

128 From this hit-list, a core set of molecules that inhibited planktonic growth of all three
129 fungi as identified by >50% growth inhibition measured by MIC were identified and
130 shortlisted. *C. albicans* was sensitive to fluconazole at concentration <0.125-0.25 μ g/ml,
131 as has been reported previously (16), while consistent with its drug resistant nature, *C.*
132 *auris* was resistant to fluconazole with MIC >16 μ g/ml (Table 2). *A. fumigatus*
133 succumbed to voriconazole at 0.25 μ g/ml, similar to previously reported anti-fungal drug
134 susceptibility studies (16). Recently, Siles *et al.* investigated the ability of NPW to
135 specifically inhibit *C. albicans* biofilms, and revealed 38 pharmacologically active agents
136 against the fungus (17). While our study also identified a number of molecules
137 individually inhibiting the three fungi, respectively (Table. 1), only the following six
138 compounds were successful at inhibiting all three organisms: chloroxin, thimerosal,
139 alexidine dihydrochloride, haloprogin, clioquinol and butenafine hydrochloride (Table 1).
140 NPW contains a number of antifungal drugs, such as imidazoles', triazoles and polyene
141 class of drugs. *C. auris* was by far the most resistant fungus, inert against the azoles

142 and polyenes in the library. The six molecules were further evaluated for their ability to
143 curtail biofilm formation as well as kill pre-formed biofilms developed by the three fungi.

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

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

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

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

186 Alexidine dihydrochloride is a bis(biguanide) in which the common 2-ethylhexyl chain
187 has been attached to each biguanide unit and the two units are linked by a 1,6-

188 hexanediamine chain (**S3A**). This compound initially identified for its anti-bacterial
189 properties, is also found as an inducer of mitochondrial dysfunction and apoptosis (11,
190 24). AXD has been proposed in several studies as an anti-plaque agent, mouthwash,
191 and with potential to be used in endodontic treatment to eliminate biofilms (19, 20, 25).
192 These reports, along with our present findings, serve as precedents for the development
193 of AXD as an anti-biofilm agent. Another bisbiguanide with structural similarity to AXD,
194 metformin, has recently been shown to have antifungal activity (and synergistic
195 potentiation of clinically used antifungal drugs) against *C. glabrata*, albeit only at
196 physiologically inapt high concentrations (26). AXD, on the other hand is active even at
197 levels as low as 0.75 µg/ml, against an array of fungal species in our current study. In
198 fact, AXD has been reported to have activity against the fungus *C. neoformans*, by
199 targeting phospholipases (27). Whether specific inhibition of fungal phospholipases is
200 the cause of AXD's antifungal activity against a spectrum of pathogenic fungi, is
201 unknown and remains to be explored in future studies.

202 **Mammalian cell cytotoxicity assays and synergy of AXD with fluconazole.**

203 Considering that AXD displayed enhanced efficacy against fungal organisms, we
204 evaluated the extent of its cell toxicity (CC50) to mammalian cells. Results showed that
205 AXD resulted in 50% killing of HUVECs and lung epithelial cells, at concentrations 5-10
206 fold higher than the MIC required to kill planktonically growing fungal pathogens (CC50
207 >7.37 µg/ml vs planktonic MIC50 of 0.73-1.5 µg/ml) (**Fig. 2A, B**). Previous studies have
208 reported similar cytotoxicity levels of AXD against various other cell lines (19, 27, 28).
209 We further tested the toxicity of AXD to a human bone-marrow derived macrophage cell
210 line to understand its effect on the immune cells. AXD displayed a slightly higher toxicity

211 to the macrophages compared to the mammalian tissue cell lines, with a CC50 of over 5
212 µg/ml (**Fig 2C**). A similar study was also done to test the impact of AXD toxicity on HL60
213 monocyte proliferation. HL60 cells stained with CFSE were treated with varying
214 concentrations of AXD, or the control PMA (phorbol 12-myristate 13-acetate) as a
215 positive stimulant that induces cellular proliferation. Inhibition of cellular proliferation
216 corresponds to toxicity, and the concentration of AXD that could prevent early cell
217 division in HL60 cells was examined. As expected cells stimulated by PMA showed
218 proliferation, while those treated with the highest dose of AXD (10 µg/ml) did not divide.
219 AXD at 5 µg/ml prevented cell division in the human bone marrow derived HL60 cells
220 (**S3B**). This level of toxicity matched the macrophage CC50 value. We note that
221 concentrations detrimental to host cells are at least 3-4 fold higher than AXD levels
222 required to inhibit planktonic cells of many different fungi, including *C. albicans*. These
223 moderately low cytotoxicity of the FDA approved drug pave the way to a potential
224 repurposing of AXD as an antifungal agent, and warrant its further development into a
225 compound with higher efficacy, bioavailability and less toxicity.

226 This inhibitory potential was further highlighted in our studies evaluating synergistic
227 action of AXD in combination with fluconazole, against mature *C. albicans* biofilms.
228 Fluconazole is completely inert against *C. albicans* biofilms, with an MIC50 of >250
229 µg/ml {this study and (7, 29, 30)}. When used together, AXD at 1.25 µg/ml, strikingly
230 reduced the MIC50 of fluconazole from >256 µg/ml to clinically relevant 1 µg/ml (**Fig**
231 **2D**), providing an FIC index of 0.42, that indicated a synergistic interaction (30). These
232 results further emphasize AXD's prospect as an anti-biofilm agent, especially due to its

233 ability to lower MICs of fluconazole, highlighting the possibility of bringing a biofilm-
234 redundant drug back into clinical use.

235 **Inhibition of biofilm *in vivo* by AXD.** Our studies showed that AXD could arrest
236 growth and kill biofilm cells formed by various *Candida* species, *C. neoformans* and *A.*
237 *fumigatus* in *in vitro* assays. We next examined the ability of AXD to decimate
238 preformed biofilms in an *in vivo* model. For this study we chose to focus on biofilm
239 formation by *C. albicans*, since a murine biofilm model has been well established in this
240 fungus and used for testing the effects of established and new antifungal agents (31).
241 The effect of the drugs on the 24 h old biofilms growing in the jugular vein catheters of
242 mice was visualized microscopically, which revealed significantly lower density of the
243 biofilms in catheters treated with AXD and caspofungin, versus the control untreated
244 catheters (**Fig 3A**). In fact, fungal CFU determination revealed that AXD inhibited 67%
245 of fungal biofilm growth and viability, compared to the control untreated biofilms (**Fig**
246 **3B**). As expected, caspofungin (an antifungal drug known to be hyperactive against *C.*
247 *albicans* biofilms) decimated >90% of the biofilm community growing within the
248 catheters. On the other hand, fluconazole (a drug with enhanced activity against
249 planktonic fungi, but with no effectiveness against biofilm cells) was found to reduce
250 biofilms by only 30% (**Fig 3B**). Overall our data shows that AXD can inhibit biofilm
251 growth *in vivo*. A better understanding of the pharmacokinetics/pharmacodynamics of
252 AXD could be invaluable in assessing its utility as a systemic antifungal drug, especially
253 in a disseminated mouse model of fungemia.

254 In summary, our HTS identified alexidine dihydrochloride to have profound activity
255 against various growth forms of fungi: planktonic, biofilm and biofilm dispersal. AXD was

256 fungicidal to a number of different pathogenic fungi including common as well as
257 emerging drug resistant pathogens. The fact that AXD retains its activity against azole
258 resistant clinical isolates indicates its potential use in recalcitrant fungal infections.
259 Importantly, AXD reduces the MIC of fluconazole-a clinically used first line antifungal
260 drug, ironically considered dispensable for biofilm treatment, thereby pointing to its
261 extended utility as an anti-biofilm combination drug. Perhaps the most intriguing activity
262 of AXD was seen against Mucorales including *Rhizopus*, a species that leads to
263 devastating infections and very poor outcomes in patients, despite conventional
264 antifungal treatment. Furthermore, the drug was also potent against those fungi that are
265 therapeutically unmanageable in clinics with current antifungal agents such as *L.*
266 *corymbifera* and *S. apiospermum*. Future studies will focus on the mechanism of action
267 of AXD at a molecular level, and evaluate its feasibility as a pan-antifungal drug to
268 combat infections in different clinical settings.

269 **Material and Methods**

270 **Strains, media and culture conditions:** The following fungal strains were used in this
271 study: *C. albicans* strain SC5314 which is a human clinical isolate recovered from a
272 patient with generalized candidiasis (32), several clinical isolates of *Candida* spp.
273 received from the Fungus Testing Laboratory at the University of Texas Health Science
274 Center at San Antonio: fluconazole-sensitive *C. albicans* CA1, CA4, and fluconazole-
275 resistant *C. albicans* CA6, CA10, *C. glabrata* fluconazole-sensitive CG1, CG3 and
276 fluconazole-resistant CG2. *C. parapsilosis* CP1, CP2, CP3, *C. neoformans* CN1, CN2,
277 CN3, and *A. fumigatus* AF1, AF2, AF3. Some *Candida* strains were also obtained from
278 the Division of Infectious Disease, Massachusetts General Hospital, Boston, MA:

279 fluconazole-resistant strains of *C. albicans* CA2, *C. parapsilosis* CP4, CP5, *C. krusei*
280 CK, and *C. tropicalis* CT2). The two *C. auris* isolates CAU-03 and CAU-09 were a kind
281 gift from Dr. Shawn Lockhart, Centers of Disease Control (CDC), and the filamentous
282 fungi including *Rhizopus delemar* 99.880 and *Rhizopus oryzae* 99.892, *L. corymbifera*
283 008049, *C. bertholletiae* 182, *M. circillenoides* 131 and *S. apiospermum* DI16-478 were
284 a part of the fungal bank at Division of Infectious Diseases, Los Angeles Biomedical
285 Research Center. All cultures were maintained by subculture on Yeast Peptone
286 Dextrose media (YPD) at 37°C and stocks of these cultures stored in 20% glycerol at -
287 80°C.

288 **HTS screening:** Screening was performed at the Molecular Screening Shared
289 Resource facility, at University of California, Los Angeles. A total of 50 μ l of 1×10^4
290 cells/ml fungal yeast cells (*C. albicans*, *C. auris*) or spores (*Aspergillus*) were
291 suspended in RPMI-1640 supplemented with L-glutamine (Cellgro), buffered with 165
292 mM morpholinepropanesulfonic acid (MOPS), and plated into individual 384-well plates
293 using an automated Multidrop 384 system (Thermo LabSystems). The New Prestwick
294 Chemical Library consisting of 1233 drugs was used to pin one compound per well at 10
295 μ M final concentration, using a Biomek FX liquid handler. Forty eight hours later the
296 plates were scanned with a Flex Station II 384 well plate reader (Molecular Devices) to
297 measure turbidity (OD600) of the wells. Molecules displaying >80% reduction in turbidity
298 compared to control non-drug treated wells (MIC80) were considered as primary “hits”.
299 Compounds commonly inhibiting all three fungal organisms were prioritized for
300 planktonic dose response assays and for their activity against biofilm growth.

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

313 **Biofilm growth and drug susceptibility testing:** Biofilms of *Candida* spp., *C.*
314 *neoformans*, and *A. fumigatus* were developed in 96-well microtiter plates, and
315 susceptibility of the biofilm cells to AXD or thimerosal was carried out as described
316 previously (33, 34). Biofilms were initiated either in the presence or absence of the
317 drugs, or the drugs were tested on 48 h pre-formed biofilms, for efficacy evaluation.
318 Inhibition of biofilm growth was measured by a standard calorimetric assay XTT that
319 measures metabolic activity of the biofilm cells (18). Absorbance at 490 nm was
320 measured using an automated plate reader. Biofilms formed by several other *Candida*
321 spp. were further studied for their susceptibility to AXD.
322 Potential of AXD for synergistic use with fluconazole against *C. albicans* biofilms was
323 investigated using a checkerboard assay, where dilutions of fluconazole (0.25 to 250

324 $\mu\text{g}/\text{ml}$) and AXD (0.3 to 2 $\mu\text{g}/\text{ml}$) were examined alone and in combination. Biofilm killing
325 was measured by XTT assay. Drug concentration associated with 50% reduction in
326 optical density compared to the no-drug control wells (EC_{50}) was determined. The
327 fractional inhibitory concentration (FIC) was then calculated as follows: $[(\text{EC}_{50} \text{ of drug A}$
328 $\text{in combination})/(\text{EC}_{50} \text{ of drug A alone})] + [(\text{EC}_{50} \text{ of drug B in combination})/(\text{EC}_{50} \text{ of drug}$
329 $\text{B alone})]$. Values of ≤ 0.5 revealed synergy, those of > 0.5 but < 2 indicated no
330 interaction, and those of > 2 were antagonistic (30).

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

339 A549 cells were purchased from the American Type Culture Collection and grown in
340 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine
341 serum. A549 cells ($1.5 \times 10^5/\text{well}$) were used to seed 96-well plates and incubated at
342 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. The medium was then
343 removed by aspiration, and the cells were washed twice with phosphate-buffered saline.
344 Treatment with AXD was conducted with DMEM supplemented with 1% FBS.

345 Different concentrations of AXD in respected media were introduced into the cell lines,
346 and incubated for 24 h at 37°C in 5% CO_2 . The extent of cellular damage to both cell

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

362 **Cytotoxicity to immune cells:** Wild-type C57Bl/6 primary bone marrow-derived
363 macrophages were cultured by plating bone marrow cells in 50 ng/ml of M-CSF
364 (Peprotech, Rocky Hill, NJ) in complete RPMI (RPMI 1640 with 2 mM L-glutamine, 10%
365 heat-inactivated fetal bovine serum, and 1% penicillin-streptomycin) for 7 days, then
366 counted and seeded at 1x10⁵ in 100 µl of complete PRMI overnight to allow for
367 adhesion.

368 To examine cytotoxicity of AXD, bone marrow-derived macrophages were incubated in
369 varying concentrations of AXD for 24h and stained with DAPI (Invitrogen, Carlsbad, CA)

370 for viability assessment using an inverted epifluorescence microscope (Olympus IX70,
371 Center Valley, PA) using 10X objective, with a X-cite 120 metal halide light source
372 (EXFO, Mississauga, ON, Canada). Percent cell viability was determine using 1 –
373 [DAPI-positive cells were divided by total cells by phase contrast] x 100.
374 AXD was also examined for its capacity to block proliferation of a human promyelocytic
375 cell line, HL-60. Cells were stained with 2 mM CFSE (Carboxyfluorescein Succinimidyl
376 ester) for 5 minutes and washed with 1X RPMI media three times. This dye is
377 commonly used to measure cell proliferation; with each cell division the amount of
378 CFSE is diluted in half, which can be observed via flow cytometry (37). After the
379 staining, the cells were counted and adjusted at cell density of 5X10⁶ cells/ml and plated
380 100 µl/well in a round bottom 96-well plate. A two-fold serially diluted AXD was added in
381 wells containing cells. The final drug concentration obtained was between 0.004 and 10
382 µg/ml. Drug-untreated and unstained cells in a number of wells were included as
383 controls. Plates were incubated at 37°C for 48 hours to allow the cell proliferation. After
384 48 hours, the cells were collected and acquired in flow cytometer. The unstained cells
385 were used to gate the CFSE positive HL60 cells. The shift in the peak of CFSE+ HL60
386 cells were considered proliferating cells.

387 ***In vivo* biofilm drug susceptibility.** A mouse central venous catheter infection model
388 was used for biofilm studies as previously described (31). These *in vivo* experiments
389 were approved by the Los Angeles Biomedical Research Institute, Harbor-UCLA-
390 IACUC. Briefly, we used catheterized 8-week old C57BL/6 male mice, purchased from
391 Charles River labs (Wilmington, MA), where the surgery was performed. The surgery
392 involves insertion of a Silastic catheter into the jugular vein of the mice. Patency is

393 tested, and the catheter is filled with heparin lock solution and plug-sealed. Following
394 receipt of the jugular vein catheterized mice, the catheters were instilled with 25 μ l of *C.*
395 *albicans* inoculum of 5×10^6 cells/ml (entire catheter volume) using a 23-gauge blunt-
396 ended needle after removal of the plug and the lock solution (the plug will be put back in
397 place after inoculation). Cells were allowed to develop biofilms for 24 h, after which the
398 catheters were treated with 3 μ g/ml AXD for 48 h. Biofilms growing in replicate mice
399 catheters were also subjected to fluconazole (250 μ g/ml) or caspofungin (0.125 μ g/ml),
400 as comparative controls. The catheters were cut laterally and imaged under a phase
401 contrast microscope to visualize the morphology of the cells growing within the
402 catheters of the individual groups. Additionally, the distal 2 cm of the catheters were into
403 small pieces, vortexed vigorously and homogenized for plating on to YPD plates for
404 viability count measurements.

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

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414 **Acknowledgment**

415 We would like to thank Dr. Nathan Wiederhold, Director, Fungus Testing Laboratory,
416 University of Texas Health Sciences Center at San Antonio, for providing us with
417 several clinical isolates of fungi used in this study. These studies were funded by a
418 grant from the National American Heart Association #16SDG30830012 to PU. ASI is
419 supported by Public Health Service grant from the National Institutes of Allergy and
420 Immunology R01 AI063503.

421 **Figure legend:**

422 **Figure 1. Inhibition of biofilm growth, *C. albicans* biofilm dispersal, and**
423 **abrogation of planktonic growth in diverse fungi by alexidine dihydrochloride**
424 **(AXD):** A) Fungal cells were allowed to form a biofilm for 48 h and treated for 24 h with
425 10 μ M AXD. Biofilm inhibition was as determined by XTT reading (OD490). B) Fungal
426 yeast cells or spores were incubated under different concentrations of AXD under
427 planktonic conditions. Inhibition of growth and filamentation of the fungi visualized by
428 phase contrast microscopy (20X magnification), at their respective AXD MIC80
429 concentrations. C) *C. albicans* planktonic hyphae (top two panels) and biofilms (bottom
430 two panels) were treated for 12 h with 150 ng/ml of AXD. AXD inhibited lateral yeast
431 production from hyphal cells and hyphal layers of biofilms, as visualized microscopically.
432 Arrows point to lateral yeasts.

433 **Figure 2. Toxicity of AXD on host cells, and on biofilm killing in combination with**
434 **fluconazole.** Different concentrations of AXD were incubated with HUVEC (A), lung

435 A549 (B), or macrophages (C) for 24 h at 37°C, for testing the CC50 of the drug to the
436 respective cell lines. (D) *C. albicans* biofilms were developed for 48 h and then treated
437 with different concentrations of AXD and fluconazole in a checkerboard format.
438 Metabolic activity of biofilm cells were measured by the XTT assay. Bright red
439 represents growth above the MIC₅₀, dull red represents growth at the MIC₅₀, and
440 black/dark red represents growth below the MIC₅₀.

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

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456 **S1:** Primary screening: MIC80 of alexidine dihydrochloride and thimerosal against pre-
457 formed biofilms of three different fungi as determined by XTT reading (OD490). Both
458 drugs kill biofilm cells at <10 μ M concentration.

459 **S2:** Inhibitory effect of AXD against pre-formed biofilms of four different fluconazole
460 resistant *Candida* spp. AXD kills preformed biofilms between 3-6 μ g.

461 **S3A:** Chemical structure of Alexidine dihydrochloride

462 **S3B:** Inhibition of early cellular division of HL60 cells by AXD
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480 **Table 1:** Table 1. Hits obtained from primary screening of the New Prestwick Chemical Library
481 against planktonic cells of *C. albicans*, *A. fumigatus* and *C. auris*
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<i>C. albicans</i>	<i>A. fumigatus</i>
Alexidine dihydrochloride	Alexidine dihydrochloride
Amphotericin B	Butenafine Hydrochloride
Antimycin A	Isoxsuprine hydrochloride
Butenafine Hydrochloride	Clioquinol
Chloroxine	Thimerosal
Ciclopirox ethanolamine	Dequalinium dichloride
Clioquinol	Pyrvinium pamoate
Clotrimazole	Halopropgin
Dequalinium dichloride	Chlorhexidine
Econazole nitrate	Voriconazole
Enilconazole	Amphotericin B
Fluconazole	Antimycin A
Flucytosine	Econazole nitrate
Halopropgin	Enilconazole
Isoconazole	Methyl benzethonium chloride
Isoxsuprine hydrochloride	Tioconazole
Itraconazole	
Ketoconazole	
Methyl benzethonium chloride	<i>C. auris</i>
Pyrvinium pamoate	Alexidine dihydrochloride
Sertaconazole nitrate	Butenafine Hydrochloride
Sulconazole nitrate	Chloroxine
Terconazole	Clioquinol
Thimerosal	Thimerosal
Thonzonium bromide	Halopropgin
Tioconazole	
Voriconazole	

483 **Table 2.** MIC of AXD against clinical isolates of different fungal species, vs fluconazole or
 484 voriconazole. Values are in $\mu\text{g/ml}$. CA=*C. albicans*, CG=*C. glabrata*, CP=*C. parapsilosis*, CK=*C.
 485 krusei*, CN=*C. neoformans*, AF=*A. fumigatus*

Isolates	Planktonic Fluconazole	Planktonic AXD (50%)	Planktonic AXD (80%)	Biofilm inhib AXD (50%)	Biofilm inhib AXD (80%)	Mature biofilm AXD (50%)	Mature biofilm AXD (80%)
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

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