1   Novel keto-alkyl-pyridinium antifungal molecules active in models of in vivo *Candida albicans*

2   vascular catheter infection and ex vivo *Candida auris* skin colonization.

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4   Running title: Novel pyridinium antifungal molecule

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Abstract

New antifungal therapies are needed for both systemic, invasive infections as well as superficial infections of mucosal and skin surfaces as well as biofilms associated with medical devices. The resistance of biofilm and biofilm-like growth phases of fungi contributes to the poor efficacy of systemic therapies to non-systemic infections. Here, we describe the identification and characterization of a novel keto-alkyl-pyridinium scaffold with broad spectrum activity (2-16 µg/mL) against medically important yeasts and moulds, including clinical isolates resistant to azoles and/or echinocandins. Furthermore, these keto-alkyl-pyridinium agents retain substantial activity against biofilm phase yeast and have direct activity against hyphal *A. fumigatus*. Although their toxicity precludes use in systemic infections, we found that the keto-alkyl-pyridinium molecules reduce *C. albicans* fungal burden in a rat model of vascular catheter infection and reduce *Candida auris* colonization in a porcine ex vivo model. These initial pre-clinical data suggest that molecules of this class may warrant further study and development.

2

Introduction

The effective treatment of human fungal infections is reliant on a relatively small set of structurally and mechanistically distinct drugs: azoles, polyenes, and echinocandin/triterpenoid glucan synthase inhibitors (1). As has been extensively discussed in the literature, this limited antifungal pharmacopeia must be expanded to meet the challenging clinical needs of patients in the 21st century (2). Two specific examples where current antifungal therapies are relatively ineffective are: 1) infections involving biofilm on medical devices and mucosal surfaces (3) and 2) decolonization of *Candida auris* from skin (4). As with antibacterial agents, fungal biofilms are highly resistant to the currently used antifungal drugs (3). Consequently, removal of *Candida* infected medical devices such as vascular catheters and prosthetic joints is the standard of care (5), whereas devices infected with some species of bacteria are frequently salvaged with antibiotic therapy alone (6).

C. auris is an emerging fungal pathogen with many unusual characteristics compared to other *Candida* species (3). For example, it is readily transmitted from one patient to another in the hospital setting (7). This is very uncommon for other fungal pathogens and is likely due to its ability to persistently colonize patient's skin as well as the inanimate surfaces in hospitals and long-term care facilities. Standard biocides used to decolonize microbes (e.g., methicillin-resistant *Staphylococcus aureus*) from patient's skin such as chlorhexidine have relatively poor activity against *C. auris* (8). Furthermore, *C. auris* is frequently resistant to at least one of the three major classes of antifungal drugs (9) and pan-resistant isolates have been reported with increasing frequency (10). As such, *C. auris* is a pernicious problem that can result in therapeutically intractable infections.

One approach to treating surface-associated fungal infections such as biofilms and skin colonization is to identify molecules that are mechanistically distinct from those used to treat systemic infections and are specifically developed for that purpose. Such molecules could be imagined to display the general properties of traditional antiseptics but with activity optimized for

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79   specific organisms or improved safety profile. Here, we describe a set of novel N-keto alkyl

80   pyridinium ions with broad spectrum antifungal activity against both planktonic and biofilm phase

81   fungi.

82   Pyridinium-based anti-septic/anti-infective molecules have been studied and developed

83   for topical and, in some instances, possible systemic use. Molecules in this class have been

84   explored for antifungal applications (11, 12). For example, the long chain N-alkylated pyridinium

85   cetylpyridinium chloride, a component of commercial mouthwashes, has in vitro activity against

86   *Candida* spp with minimum inhibitory concentrations (MIC) of 2-6 µg/mL in modified CLSI

87   conditions (13). A recent repurposing screen found that the anti-parasitic pyridinium-class drug

88   pyrvinium pamoate is active against *C. auris* (MIC 2 µg/mL, ref. 14). Bis(pyridinium) molecules

89   with variously sized alkyl or aryl linkers have also been shown to have antifungal activity (11,

90   15). One study showed that the bis(pyridinium) compounds induced less red blood cell lysis

91   than similar bis(quaternary-ammonium) compounds (15).

92   The series characterized in this report are mono-pyridinium ions with aryl-alkyl ketone

93   substituents at the N atom of the pyridinium ring (Fig. 1A). The molecules are comparable in

94   activity to the commercially used, cetylpyridinium chloride and pyrvinium pamoate and have

95   activity in an in vivo rat model of *C. albicans* vascular catheter infection as well as in an ex vivo

96   model of *C. auris* skin colonization.

97

98   **Results**

99   **Aryl methyl keto-*N*-alkylated pyridinium molecules have broad spectrum antifungal**

100   **activity.**

101   A set of small molecule libraries were screened for compounds against *Aspergillus*

102   *fumigatus* using recently developed high throughput screening assay applicable for this

103   filamentous mould (16). A molecule with the aryl methyl keto-N-alkylated pyridinium (**k**eto-**a**lkyl

104   **p**yridinium, KAP) scaffold (Fig. 1A) was identified a hit in the screen. A set of analogs of this

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105 initial hit were prepared and tested, leading to two derivatives with similar activity profiles

106 (compounds **A** and **B**, Fig. 1A); **C** was used a control as it is inactive but has a very similar

107 chemical structure compared to the active derivatives. Although >30 KAP derivatives were

108 prepared, no clear structure-activity relationships (SAR) could be identified to explain the

109 variation in antifungal properties of this series (see Fig. S1 for general synthesis approach).

110 Pyridinium cations are widely believed to target membranes as part of their mechanism of action

111 and a non-protein target could explain this difficult-to-interpret SAR (13, 17). Although other

112 potential targets have also been proposed, a biochemically or genetically confirmed alternative

113 target to the membrane has not been reported (12, 14). It is, however, interesting that small

114 changes in substitution patterns in comparing A/B to C (Fig. 1A) lead to dramatic changes in

115 antifungal activity without clearly identifiable changes in physicochemical properties. These

116 observations suggest that the molecules have structurally specific interactions with a target and

117 imply that interaction with membranes is not solely due to their amphipathic properties.

118 The scope of the antifungal activity for compounds A and B was explored by CLSI

119 methods for a range of fungal pathogens including yeasts and moulds (Table 1). In general, A/B

120 are more consistently active against yeasts compared to moulds (*A. fumigatus*, *A. terreus*, and

121 *F. oxysporum*). Although the MIC for some isolates of *A. fumigatus* was similar to those for

122 yeast, significant strain-to-strain variability in activity toward *Aspergillus* was observed. We also

123 tested A/B against two fluconazole-resistant *C. albicans* strains (TWO15/17, ref. 17) that have

124 increased expression of efflux pumps. We no difference in susceptibility compared to the

125 reference strain SC5314 (Table 1). Furthermore, compounds A and B were both active against

126 drug susceptible (0381) and multi-drug resistant (0390) *C. auris* isolates (Table 1).

127 Since pyridinium cations such as cetylpyridinium chloride also have activity against

128 bacteria, we tested compounds A and B against the Gram-positive bacteria, *Staphylococcus*

129 *aureus*, and the Gram-negative bacteria, *Pseudomonas aeruginosa*. Compared to

130 cetylpyridinium choloride, compounds A and B were ~32-fold less active against *S aureus* but

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131 were more active against *P. aeruginosa* (32 µg/mL vs 250 µg/mL reported in ref 17). In

132 contrast, the activity of A/B were within 2-fold of the MICs reported for cetylpyridinium chloride

133 against *C. albicans* and pyrvinium pamoate against *C. auris*.

134 To determine if compound interacted with clinically used antifungal drugs, we performed

135 checkerboard assays and determined fractional inhibitory concentration indices. Compound B

136 showed additive interactions with both caspofungin and amphotericin B against *C. albicans* but

137 showed a complex interaction with fluconazole. The FICI for compound B and fluconazole is

138 8.25 due to the 8-fold increase in fluconazole concentration in the combination; however, the

139 concentration of compound B in at FIC is reduced by 4-fold relative to compound A as a single

140 agent. Interestingly, Edlind et al. also observed that cetylpyridinium chloride showed antagonism

141 with fluconazole and induced expression of the efflux pumps *CDR1* and *CDR2* (13).

142 In contrast to the antagonistic interaction of compound B with fluconazole in *C. albicans*,

143 voriconazole, the gold standard therapy for aspergillosis, showed an additive interaction.

144 Indeed, the activity of compound B was improved 8-fold in combination with voriconazole at ½

145 MIC. These data indicate that the induction of relative azole resistance in *C. albicans* by B does

146 not occur with *A. fumigatus*. Efflux pump mediated resistance to azoles is not as prevalent in *A.*

147 *fumigatus* compared to *C. albicans* and, thus, the mechanisms of efflux pump induction and

148 function are not as well defined.

149

150 **KAPs are active against *Candida* biofilms and *A. fumigatus* hyphae**

151 In general, pyridinium cations are too toxic for systemic administration because many

152 examples of this class directly lyse red blood cells (15). Consistent with those expectations,

153 compound A and B cause significant red blood cell lysis (LD$_{50}$ A: 26.9 µg/mL; B: 78.6 µg/mL;

154 and C > 1000 µg/mL, Fig. S1) while compound C, which has no antifungal activity also had no

155 activity against red blood cells. This observation provides implicit support for the membrane as

156 an important target of these molecules. Both A and B also showed toxicity against the human

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157   cell line HegG2 with LD_{50} of 9 µg/mL and 20 µg/mL using cell lysis and metabolic activity

158   assays, respectively (Fig. S1). Consequently, the KAPs, like other quaternary nitrogen anti-

159   infectives, are likely to be suitable for topical or other non-systemic applications and not feasible

160   for systemic therapy.

161   As discussed above, many anti-infectives have dramatically reduced activity against

162   biofilm phase organisms compared to the planktonic growth phase of standard CLSI activity

163   assays. To test the activity of the compounds against fungal biofilms, we generated a 24 hr

164   biofilm of *Candida albicans* and then treated with a dilution series of the compounds for an

165   additional 24 hr. The metabolic activity of the biofilms was then assayed using the standard XXT

166   reduction assay (19). Consistent with planktonic results, compound A was active (Fig. 2A, IC_{50} =

167   18.4 µg/mL) while C was not. The biofilm activity of compound A was reduced by 4-fold relative

168   to planktonic growth (MIC 4 µg/mL, Table 1). Interestingly, the activity of compound A was

169   slightly higher against *C. auris* biofilms (Fig. 2B IC_{50} = 5 µg/mL) relative to planktonic growth

170   (MIC 8 µg/mL, Table 1).

171   CLSI testing of antifungal activity of moulds is based on inoculation with conidia and,

172   therefore, measures inhibition of germination (20). For example, voriconazole is the gold

173   standard for treatment of pulmonary infection and inhibits germination. The most important *A.*

174   *fumigatus* infection that requires non-systemic therapy is fungal keratitis (21). At the time of

175   clinical presentation, *A. fumigatus* is exclusively in the hyphal form. We, therefore, were

176   interested to determine if compound A or B was active against *A. fumigatus* hyphae. *A.*

177   *fumigatus* CEA10 was incubated for 24 hr prior to exposure to a dilution series of compound B

178   or C for an additional 24 hr. The metabolic activity of the cultures was determined using

179   resazurin as previously described (17). The IC_{50} (3.9 µg/mL, Fig. 2C) of compound B against

180   this strain was slightly lower than its MIC (8 µg/mL, Table 1) determined by CLSI methods. To

181   further characterize the mode of action of the KAPs against *A. fumigatus* hyphae, we treated

182   CEA10 with compound B for 24 hr and then stained the cells with propidium iodide, a dye that is

183 excluded from cells with intact membranes (Fig. 2D). Whereas cells treated with DMSO control

184 showed essentially no staining, B-treated cells showed uniform uptake of dye, indicating loss of

185 membrane integrity. Taken together, these data indicate that KAPs directly kill hyphal stage *A.*

186 *fumigatus.*

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188 **KAP A is efficacious in an in vivo model of *C. albicans* venous catheter infection.**

189 One potential approach to managing central venous catheter infections is to treat the

190 lumen of the catheter with an anti-infective solution that is locked within the catheter and not

191 introduced into the patient (22). This strategy is most often applied to catheters infected with

192 bacteria but has been proposed for fungal infections as well (5, 6). To test the efficacy of KAPs

193 in this setting, a rat model of *C. albicans* venous catheter infection was employed (23). The

194 catheter was infected with *C. albicans* SC5314 and, on post-infection day 1, treated with A (8

195 µg/mL solution within catheter) or vehicle. After 24 hr, the catheter was removed and processed

196 for fungal burden and scanning electron microscopy. Compound A-treated catheters showed >

197 1 $\log_{10}$ reduction in fungal burden relative to vehicle-treated catheters (Fig. 3A). Consistent with

198 these results, the treated catheters showed dramatic in the extent of remaining biofilm. Thus,

199 compound A is able to disrupt a pre-formed *C. albicans* biofilm in vitro and in vivo.

200

201 **KAP A reduces *C. auris* colonization of skin in an ex vivo porcine model**

202 The ability of *C. auris* to colonize human skin is likely associated with its persistence in

203 infected patients and its ability to transmit from person-to-person (7). Because compound A

204 showed comparable activity against *C. auris* during planktonic and inanimate-substrate biofilm

205 growth, we tested its activity in an ex vivo porcine model of *C. auris* skin colonization (24).

206 Consistent with these in vitro data, compound A reduced the fungal burden of the colonized

207 porcine skin by ~1 $\log_{10}$ at a concentration of 8 µg/mL (Fig. 4). The fungal burden was not

208 reduced further at 16 µg/mL of compound A, suggesting that absorption/adsorption or

209   physicochemical factors may limit efficacy. The activity of compound A was greater than that of

210   2% chlorhexidine (~0.5 $\log_{10}$ reduction, ref. 8) and comparable to the synergistic activity of 2%

211   chlorhexidine/70% isopropanol (1.0 $\log_{10}$ reduction, ref. 8). We note that, due to limitations in

212   compound A availability, that the data for skin treated with compound A was collected 24 hr

213   after a single treatment while the studies with chlorhexidine and 70% isopropanol were based

214   on a 72 hr experiment with three daily applications of study compounds. These experimental

215   distinctions notwithstanding, compound A shows promising activity against *C. auris* and is likely

216   at least as effective, and possibly more effective, than 2% chlorhexidine.

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Discussion

219   Although new therapeutic options for systemic therapy of fungal infections are sorely

220   needed (2), agents with promise in the treatment of other types of fungal infections, particularly

221   those involving biofilm and biofilm-like growth phases, would also be quite valuable (3,4). Here,

222   we have characterized the activity of a structurally novel series of pyridinium cation-based

223   antifungal molecules and demonstrate their potential for the treatment of non-systemic fungal

224   infections. Compounds A and B show comparable or improved activity relative to other cationic

225   nitrogen-based anti-infectives/anti-septics such as chlorhexidine and cetylpyridinium chloride

226   against fungal pathogens (8, 13, 14). In addition, A and B have similar activity to the pyridinium-

227   based drug pyrvinium pamoate against *C. auris* against in vivo biofilms (14), further supporting

228   the utility of this class of molecule against this important emerging and drug-resistant pathogen.

229   Our in vivo and ex vivo experiments provide compelling proof-of-principle data for the

230   potential use of these scaffolds in the setting of antifungal lock therapy for intravascular

231   catheters and as possible disinfectants for *C. auris* skin colonization. With respect to the latter,

232   compound A is more active than the widely studied chlorhexidine (8) which was shown to have

233   activity in a mouse model of colonization (25). Since these compounds are variations on

234   previously validated chemical structures with application to topical and other non-systemic uses,

235   a strong premise exists for their potential development. Additional potential applications for

236   which our data provide support are oropharyngeal candidiasis and fungal keratitis. The latter

237   infection is a globally important cause of blindness for which there is no generally effective

238   medical therapy (21). Compounds A and B have reasonable activity against the two most

239   common etiologic agents of fungal keratitis: *A fumigatus* and *Fusarium* spp (Table 1). The direct

240   activity against hypha is an important feature of this series but additional optimization will be

241   needed to improve the consistency of activity and therapeutic index to become useful for fungal

242   keratitis.

243   The mechanism of action for quaternary nitrogen-based antifungals such as the KAPs is

244   likely to involve, at least in part, the direct disruption of membrane structures (17). Recent work

245   by Sim et al. suggested that pyrvinium pamoate interferes with mitochondrial function (14) and

246   mode of action studies of the cationic amidine antifungal candidate T-2307 as well as other

247   cationic ammonium antifungals also showed that mitochondrial disruption contributes to their

248   activity (12, 26). A hallmark of mitochondrial disruption in *C. albicans* is an inability to grow on

249   non-fermentable carbon sources such as glycerol. Indeed, the MIC of T-2307 decreases

250   dramatically when the cells are grown on 2% glycerol compared to 2% dextrose (26). We,

251   however, did not detect a change in MIC for compounds A or B when tested in the same

252   medium with glycerol instead of glucose as carbon source, indicating that mitochondrial

253   disruption is unlikely to be a major mode of action (data not shown). Attempts to generate

254   resistant mutants by serial passaging were also unsuccessful which further suggests either

255   multiple targets or non-protein targets such as the membrane are likely to contribute to the

256   antifungal activity of the KAPs.

257   It is interesting that compound A that, like cetylpyridinium chloride, is antagonistic with

258   fluconazole in *C. albicans* in checkerboard assays. Edlind et al. showed that cetylpyrdinium

259   chloride induces the expression of the efflux pumps *CDR1* and *CDR2* but that deletion of these

260   genes has no effect on its activity (13). Compounds A and B are active as reference strains

261   against fluconazole-resistant clinical strains shown to have increased expression of efflux

262   pumps (Table 1, ref. 18). Thus, it seems that both types of pyridinium based antifungal

263   molecules alter expression of fluconazole pumps without being susceptible to their effects. As

264   such, these observations imply that they have similar mechanisms of action that are best

265   attributed to membrane disruption.

266   In summary, we provide in vitro and in vivo data suggesting that the KAP-type pyridinium

267   cation molecules may be promising new agents for the non-systemic treatment of fungal

268   infections.

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292 **Materials and methods**

293 **Strains, media, reagents and instrumentation.** All yeast strains were maintained on YPD

294 from 25% glycerol stocks stored at -80°C. All *A. fumigatus* strains were maintained on glucose

295 minimal media (GMM; (33) from 25% glycerol stocks stored at -80°C. *A. fumigatus* CFF clinical

296 isolates were received from Dr. Robert Cramer (Dartmouth College), SPF98 was received from

297 Dr. W. Scott Moye-Rowley (University of Iowa), *C. neoformans* DUMC clinical isolate series

298 were received from Dr. John Perfect (Duke University). Reagents were purchased as at least

299 reagent grade from Aldrich, Acros or Alfa Aesar unless otherwise specified and used without

300 further purification. Solvents were purchased from Fischer Scientific (Pittsburgh, PA) and were

301 either used as purchased or redistilled with an appropriate drying agent. Compounds used for

302 structure−activity studies were synthesized according to methods described below, and all the

303 compounds were identified to be least 95% pure using HPLC. Analytical TLC was performed

304 using precoated Silica G TLC Plates, w/UV254, aluminum backed, purchased from Sorbtech

305 (Norcross, GA) and visualized using UV light. Flash chromatography was carried out using with

306 a Biotage Isolera One (Charlotte, NC) system using the specified solvent. Microwave reactions

307 were performed using Biotage Initiator+ (Charlotte, NC). Purity analysis were performed on an

308 Agilent 1100 HPLC utilizing a C-18 column (Waters Nova-Pak; 3.9 x 100 mm) with the following

309 method: Solvent A = H_2O (0.1% TFA), Solvent B = Acetonitrile; 0 to 20 min, (10 to 90% B), 20 to

310 25 min (90 to 10% B); detection was set at two wavelengths (245 and 280 nm). Purity of all final

311 compounds was above 95%.   All final compounds were analyzed by high resolution MS

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312   (HRMS) using a Bruker Maxis Plus Quadrupole Time-of-Flight (QTOF or QqTOF). [1]H and [13]C

313   NMR were recorded on either a BrukerAvance III 500 outfitted with a 5mm BBFO Z-gradient

314   probe or a Avance III 300 instrument is equipped with a 5 mm BBFO probe. The chemical shifts

315   are expressed in parts per million (ppm) using suitable deuterated NMR solvents.

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317   **Dichlorodiphenylmethane (1).**

318   To a round flask was weighed benzophenone (1eq) and PCl_5 (1.5eq). The reaction was refluxed

319   for 2 h at 150°C. After 2 hours, the reaction was cooled, 40 mL of DCM was added to the

320   resultant solution followed by transferring to 250 mL Erlenmeyer flask. The resultant solution

321   was cooled to 0°C and 40 mL of distilled water was added. The resultant solution was

322   transferred to a separatory funnel, the DCM layer was separated, washed twice with 40 mL

323   distilled water, and dried with anhydrous sodium sulfate. The DCM solvent was evaporated in

324   rotary evaporator to give corresponding dichloride. The crude product was immediately used for

325   the next step without purification.

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327   **2-chloro-1-(2,2-diphenylbenzo[d][1,3]dioxol-5-yl)ethenone (2)**

328   To a round flask was weighed dichlorodiphenylmethane (1 g, 4.2 mmol) and 2-chloro-1-(3, 4-

329   dihydroxyphenyl) ethanone (0.79 g, 4.2 mmol) under nitrogen. The reaction was refluxed at

330   180°C for 30mins under nitrogen. After 30 mins. The crude product was transferred to silica gel

331   chromatography with linear gradient of 2 - 10% (EtOAc/Hexane). The fractions corresponding to

332   the desired product were collected and solvent evaporated using rotatory evaporator to give

333   yellow viscous liquid as the desired product which turned to a solid upon dryness. Yield; 0.7 g,

334   47%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.69 (m, 4H), 7.61-7.57 (m, 2H), 7.44 (m, 6H), 6.98 (d, $J =$

335   7.9 Hz, 1H), 4.63 (s, 2H).^{13}C NMR (75 MHz, $CDCl_3$), 189.26, 152.05, 148.05, 139.46, 129.56,

336   129.01, 128.41, 125.14, 118.58, 108.52, 108.38, 45.84

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338 **4-acetamido-1-(2-(2,2-diphenylbenzo[d][1,3]dioxol-5-yl)-2-oxoethyl)pyridin-1-ium chloride**

339 **(3).** To a round flask containing 2-chloro-1-(2,2-diphenylbenzo[d][1,3]dioxol-5-yl)ethenone (0.34

340 0.97 mmol) dissolved in acetonitrile (10 mL) was weighed the 4-aminopyridine (0.09 g, 0.97

341 mmol). The reaction was refluxed for 2hrs at 100°C in which precipitate was formed. After 2 hrs,

342 the solvent was evaporated using rotatory evaporator and the gummy solid precipitated with

343 10% Hexane in DCM, filtered and dried to give a powdered solid product in form of the chloride

344 salt. HPLC purity; 98%. Yield;0.38 g, 88%, ^{1}H NMR (300 MHz, DMSO) δ 12.12 (s, 1H), 8.65 (d,

345 J = 6.9 Hz, 2H), 8.22 (d, J = 6.9 Hz, 2H), 7.74 (d, J = 8.3 Hz, 2H), 7.68 (s, 1H), 7.57-7.55 (m,

346 4H), 7.48-7.32 (m 6H), 7.32 (d, J = 8.2 Hz, 1H), 6.21 (s, 2H), 2.27 (s, 3H).^{13}C NMR (75 MHz,

347 DMSO), 189.88, 171.71, 152.87, 151.87, 147.59, 146.99, 139.30, 130.21, 129.18, 129.00,

348 126.25, 125.70, 118.36, 114.77, 109.56, 108.65, 64.75, 25.06, HRMS for [M+H]$^+$ calculated;

349 451.1652, found; 451.1658

350

351 **Planktonic growth phase antifungal activity assays.** Minimum inhibitory concentrations were

352 determined using CLSI guidelines (16, 20). All yeasts were cultured overnight in 3 mL YPD at

353 30°C, then washed twice in sterile PBS. Two-fold serial dilutions of each compound were

354 prepared in RMPI+MOPS pH 7 (Gibco RPMI 1640 with L-glutamine [11875-093] and 0.165M

355 MOPS), then 1 x 10^3 cells were added per well. Plates were incubated at 37°C for 24 h (*C.*

356 *albicans* and *S. cerevisiae*) or 72 h (*C. neoformans*). For *A. fumigatus* and *F. oxysporum* wells

357 were inoculated with 1.25 x 10^4 conidia. Plates were incubated at 37°C for 48 h for *A. fumigatus*

358 and 72 h for *F. oxysporum*.

359

360 **Biofilm phase antifungal activity assays.** These assays were performed as previously

361 described (17, 19). *C. albicans* and *C. auris*, overnight cultures were washed in sterile PBS and

362 adjusted to 1x10^6 CFU/mL in RPMI+MOPS pH 7. 100μL cells were added to each well and

363 incubated at 37°C for 24 h. A two-fold dilution series of the compounds was prepared in RPMI +

364 MOPS with equal vehicle concentrations. Media was removed and biofilms were gently washed

365 with PBS, then 200µL of each drug dilution was added to wells and plates were incubated at

366 37°C for an additional 24 h. Media was removed and biofilms were gently washed with PBS,

367 then 100 µL of XTT solution (0.83 mg/mL XTT sodium salt [Sigma, Cat# X4626] + 32 µg/mL

368 PMS [Sigma, Cat# P9625] in PBS) was added to each well. Plates were incubated at 37°C for

369 30 min, then the absorbance was measured at 490 nm.

370

371 **Red blood cell lysis assay.** Hemolysis assays were performed with defibrinated sheep's blood

372 (Lampire, Cat# 7239001). Blood was washed three times with PBS then resuspended to ~50%

373 hematocrit in PBS. A two-fold dilution series of the compounds in 200µL PBS with equal DMSO

374 concentration across the series, then red blood cells were added with a final concentration of

375 2% hematocrit. Cells were incubated at RT for 2 h in a v-bottom microtiter plate, then plates

376 were spun down and supernatant was transferred to a flat bottom microtiter plate for

377 absorbance measurement at 570 nm. Compounds were tested in technical triplicate in at least

378 two independent assays performed on different days.

379

380 **Mammalian cell culture toxicity assay.** HepG2 cells (ATCC, #) were maintained and cultured

381 in HepG2 media (DMEM (Gibco, Cat#11965-092) with 5% FBS, 20 mM Glutamine, and

382 Penicillin/Streptomycin) at 37°C with 5% CO_2. Cells were seeded in 96-well plates at a density

383 of 1.25 x 10^4 cells/well and incubated overnight at 37°C with 5% CO_2, then media was removed

384 and replaced with media containing a two-fold dilution series of compounds with equal DMSO

385 concentrations across all wells including no compound controls. Cells were incubated for an

386 additional 24 h. Supernatant was removed and used to quantify lactate dehydrogenase release

387 using the CyQuant LDH assay kit (Invitrogen, Cat#C20300) following the manufacturer's

388   directions. LDH signal was normalized to a max lysis control. The remaining cells were then

389   used to quantify cellular metabolism by XTT. Briefly, cells were washed, then 0.9 mg/mL XTT

390   (Sigma, CAT#X4626) + 320 µg/mL PMS (Phenothiazine methosulfate; Sigma, Cat# P9625) in

391   HepG2 media was added to each well. Plates were incubated at 37°C, with 5% CO_2 for 2 h,

392   then absorbance was measured at 490 nm.

393

394   **Rat model of *Candida albicans* vascular catheter infection.** *C. albicans* biofilm growth during

395   infection of implanted medical devices was measured using an external jugular-vein, rat-

396   catheter infection model (23). Briefly, a 1 x 10^6 cells/ml inoculum for each strain or strain

397   combination was allowed to grow on an internal jugular catheter placed in a pathogen-free

398   female rat (16-week old, 400 g) for 24 h. After this period, the catheter volumes were removed

399   and the catheters were flushed with 0.9% NaCl. The biofilms were dislodged by sonication and

400   vortexing. Viable cell counts were determined by dilution plating. Three replicates were

401   performed for each strain.

402   Scanning electron microscopy of catheter biofilms. After a 24 h biofilm formation phase,

403   the devices were removed, sectioned to expose the intraluminal surface, and processed for

404   SEM imaging. Briefly, one milliliter fixative (4% formaldehyde and 1% glutaraldehyde in PBS)

405   was added to each catheter tube and tubes were fixed at 4 °C overnight. Catheters were then

406   washed with PBS prior to incubation in 1% OsO4 for 30 min. Samples were then serially

407   dehydrated in ethanol (30–100%). Critical point drying was used to completely dehydrate the

408   samples prior to palladium–gold coating. Samples were imaged on a SEM LEO 1530, with

409   Adobe Photoshop 2022 (v. 23.2.2) used for image compilation.

410

411   **Porcine skin model of *Candida auris* colonization.** The collection of porcine skin samples

412   was conducted under protocols approved by the University of Wisconsin–Madison Institutional

413 Animal Care and Use Committee in accordance with published National Institutes of Health

414 (NIH) and United States Department of Agriculture (USDA) guidelines. Excised skin was

415 cleaned and shaved as described previously (24). Full-thickness samples were obtained by 12

416 mm punch and placed in 12-well plates containing 3 mL Dulbecco's Modified Eagle Medium

417 (DMEM) (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS)

418 (Atlanta biologicals, Lawrenceville, GA, USA), penicillin (1,000 U/mL), and streptomycin (1

419 mg/mL) (Corning, Manassas, VA, USA) for 6 hours. Tissues were rinsed in DBPS and moved to

420 semi-solid media (6:4 ratio of 1% agarose (BIO-RAD, Hercules, CA, USA) in DPBS:DMEM with

421 10% FBS). Paraffin wax was applied around the edge of the skin and 10 µL *C. auris* suspended

422 in synthetic sweat medium at 10^7 cells/mL was applied. Samples were incubated at 24 h and

423 compound A (8 µg/mL or 16 µg/mL) in synthetic sweat medium was applied. After 24 h a sterile

424 swab was used to remove compound from skin surface. Samples were vortexed in DPBS and

425 plated on YPD + chloramphenicol to assess viable burden.

426

Acknowledgements

433

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512   **Figure Legends**

513   **Figure 1. Ketoalkylpyridinium compounds with aryl substitutions have tunable antifungal**

514   **activity. A.** The pharmacore and R_1 substitutions for compounds A, B and C with minimum

515   inhibitory concentrations (MIC; μg/mL) for *C. albicans* SC5314, *C. auris* 0381, and *A. fumigatus*

516   CEA10. **B**. Fractional inhibitory concentration indices (FICI) of B with clinical antifungals. FICIs

517 were calculated using the MIC (µg/mL) of each drug alone (MIC_A) and in combination (MIC_C)

518 based on at least two independent checkboard assays performed on separate days.

519 Interactions are defined as: $0.5 \geq$ FICI = synergy; $0.5 <$ FICI > 2 = additive; $2 <$ FICI $> 4 =$

520 indifferent; FICI ≥ 4 = antagonism.

521

522 **Figure 2. KAPs are active against fungal biofilms.** A. Metabolic activity of *A. fumigatus*

523 CEA10 biofilms (measured using resazurin) treated with increasing concentrations B or C for 24

524 hours. B. Propidium Iodide (PI) staining of *A. fumigatus* CEA10 hyphal cells treated with

525 16µg/mL B for 24 hours. Images were acquired using the same exposure and magnification for

526 all samples. Representative images from two independent experiments. Metabolic activity of

527 established C*. albicans* SC5314 (**C**) or *C. auris* 0381 (**D**) biofilms treated with **A** or **C** for 24

528 hours. For all graphs, data represent mean and SEM of three biological replicates and are

529 normalized to untreated controls. IC_{50} curves and values were calculated using GraphPad Prism

530 9.

531

532 **Figure 3. KAPs are efficacious in a rat model of *C. albicans* infection of a vascular**

533 **catheter. A**. Colony forming units (CFU) of *C. albicans* from central venous catheters places in

534 rats and treated with vehicle or **A** (8 µg/mL). Fungal burden data are shown as the mean and

535 SEM of log-transformed CFU values; n=3 rats per group. **$p<0.0001$ by unpaired t-test of log-

536 transformed values. **B**. Scanning electron micrographs of catheters treated with vehicle or 8

537 µg/mL **A**. Scale bar = 10 µm.

538

539 **Figure 4. KAP A reduces *C. auris* colonization of skin in an ex vivo porcine model.** CFUs

540 of *C. auris* on porcine skin treated with vehicle or **A** (8 µg/mL or 16 µg/mL) for 24 h. Data

21

541   represent mean and SEM of log-transformed values; n=12 per group. **p<0.0001 by unpaired t-

542   test of log-transformed values.

543

544   **Figure S1. Chemical synthesis of compound A.** Reaction conditions: (a) PCl_5 (1.5 eq), 2 h,

545   reflux 150°(b) 30 min, 180°C, reflux (c) 2 h, 100°C acetonitrile reflux.

546

547   **Figure S2. Red blood cell and mammalian cell culture toxicity of KAP compounds. A**.

548   Hemolysis of commercial red blood cells (RBC) treated for 2 h with increasing concentrations of

549   KAPs normalized to max lysis by triton-x. Mean and SD of technical triplicates. Representative

550   data from three independent experiments performed on different days are shown. Toxicity

551   against HepG2 cells using LDH release (**B**) and XTT (**C**) assays. Cells were treated with the

552   indicated concentration series of **A** for 24 h. Mean and SD of technical triplicates. Data are

553   representative of two independent experiments performed on different days.

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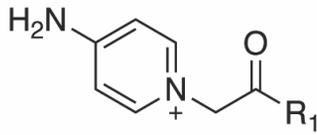
Figure 1

A

Compound	R₁	C. albicans MIC	C. auris MIC	A. fumigatus MIC
A		4	8	32
B		8	16	8
C		>128	>128	>128
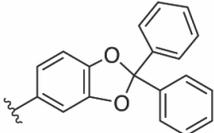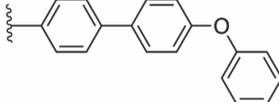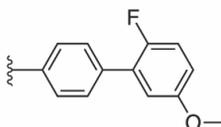
B

Species	Clinical antifungal	B		Clinical antifungal		FICI
		MIC$_A$	MIC$_C$	MIC$_A$	MIC$_C$	
C. albicans	Fluconazole	16	4	0.125	1	**8.25**
C. albicans	Amphotericin B	4	1	0.5	0.25	**0.75**
A. fumigatus	Voriconazole	16	2	0.25	0.125	**0.625**

Figure 2
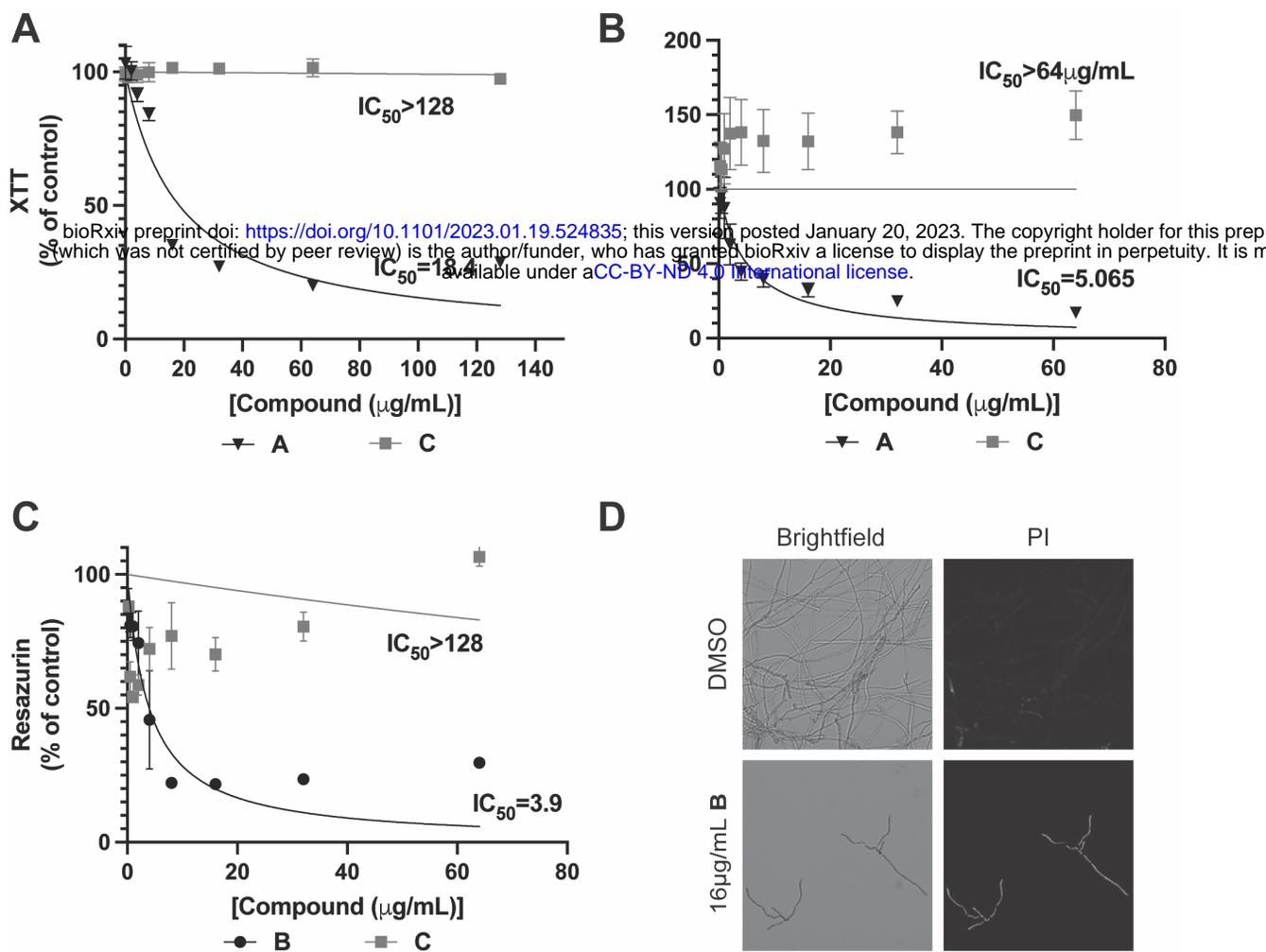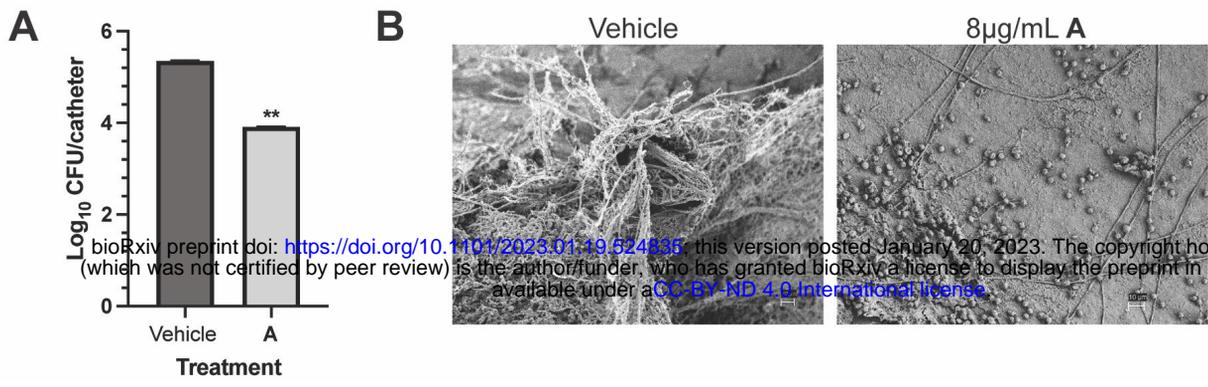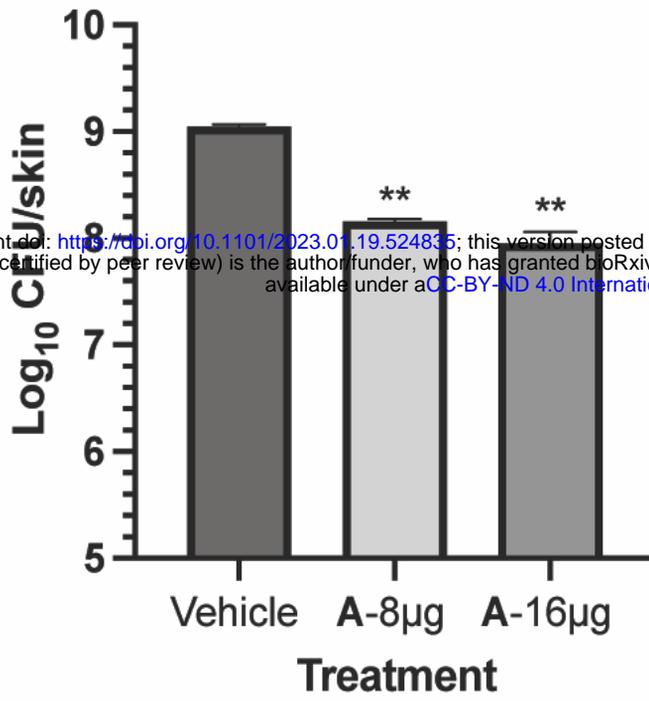
A

XTT (% of control)

IC$_{50}$>128

IC$_{50}$=18.4

[Compound (μg/mL)]

▼ A   ■ C

B

IC$_{50}$>64μg/mL

IC$_{50}$=5.065

[Compound (μg/mL)]

▼ A   ■ C

C

Resazurin (% of control)

IC$_{50}$>128

IC$_{50}$=3.9

[Compound (μg/mL)]

● B   ■ C

D

Brightfield   PI

DMSO

16μg/mL B

Figure 3

A

B

Vehicle

8µg/mL **A**

Figure 4

Table 1

Species	Strain	A MIC	B MIC
A. fumigatus	CEA10	32	8
	AF293	2	1
	SPF98	64	16
	Cas9	32	16
	CFF S014	8	NT
	CFF S008	64	NT
	CFF S007	64	NT
	CFF S001	64	NT
	CFF S029	4	NT
	CFF S003	4	NT
A. terreus	CFF S010	64	NT
	CFF S023	2-4	NT
C. albicans	SC5314	4	8
	TWO #15	4	4
	TWO #17	4	4
C. glabrata	KK2001	4	1
C. auris	0381	8	16
	0390	2	4
C. neoformans	H99	4	2
	DUMC 118.00	4	2
	DUMC 122.01	4	4
F. oxysporum	DUMC Fo	16	16
P. aeruginosa	PAO1	64	32
S. aureus	Newman	32	16

Table 1. Minimum inhibitory concentration of KAP compounds against medically important fungi and bacteria. Minimum inhibitory concentrations (MIC; µg/mL) were measured using standard CLSI broth microdilution assays. Reported MICs are representative of at least two independent assays performed on different days. NT = not tested.

Figure S1

A

IC$_{50}$=26.9

▼ A
● B
■ C

IC$_{50}$=73.6

IC$_{50}$>1000

% RBC lysis

[KAP (μg/mL)]

B

LDH Cytotoxicity (% Max lysis)

LD$_{50}$=9.18

[A (μg/mL)]

C

XTT (% of control)

IC$_{50}$=20

[A (μg/mL)]