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1 Synergistic Activity of Minocycline and Rifampin in Combination with Antifungal Drugs against
2 *Candida auris*
3
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15 ABSTRACT

16 *Candida auris* is an emerging multidrug-resistant fungal pathogen that spreads readily in
17 healthcare settings and has caused numerous hospital outbreaks. Very few treatment options
18 exist for *C. auris* infections. We evaluated the activity of all two-drug combinations of three
19 antifungal agents (amphotericin B, caspofungin, and voriconazole) and two antibacterial agents
20 (minocycline and rifampin) against a collection of 10 *C. auris* isolates using an automated, inkjet
21 printer-assisted checkerboard array method. Three antibacterial-antifungal combinations
22 (amphotericin B plus rifampin, amphotericin B plus minocycline, and caspofungin plus
23 minocycline) demonstrated synergistic activity by checkerboard array against ≥90% of strains.
24 The two amphotericin B-containing combinations were also synergistic using the time-kill
25 synergy testing method. Our results suggest that combinations of antifungal and antibacterial
26 agents may provide a promising avenue for treatment of this multidrug-resistant pathogen.
27

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28 INTRODUCTION

29 The pathogenic yeast *Candida auris*, first identified in the external ear canal drainage of
30 a woman in Japan in 2009 (1), was classified by the U.S. Centers for Disease Control and
31 Prevention as one of the most urgent antibiotic resistance threats in 2019 (2). At least 4 distinct
32 clades of the yeast appear to have emerged nearly simultaneously on 3 different continents
33 around the time the first isolate was recognized (3). More recently, invasive *C. auris* infections
34 have been observed as a complication of critical SARS-CoV-2 disease (4–6). *C. auris* is most
35 commonly reported as a cause of bloodstream infections; patients with central venous
36 catheters and recent surgical procedures appear to be at particularly high risk (7). Compared to
37 other *Candida* species, *C. auris* is notable for its propensity to spread rapidly within healthcare
38 settings, for a high rate of mortality in infected patients, and for frequent resistance to multiple
39 antifungal drugs (8, 9).

40 The number of drugs available to treat even the most susceptible of fungal pathogens is
41 small, with only three classes of systemic antifungal agents in general use: azoles,
42 echinocandins, and the polyene amphotericin B (AMB) (10). Individual patient factors, such as
43 allergies, vulnerabilities to side effects, drug-drug interactions, and the need for penetration
44 into specific tissue sites frequently further constrain the choice of agents that can be safely and
45 effectively used; resistance to any of these agents may reduce practical treatment options to
46 few or none. In this context, the resistance profiles observed among *C. auris* isolates are
47 particularly alarming: nearly all isolates are resistant to fluconazole, and some also demonstrate
48 resistance to echinocandins and AMB (7, 11). Isolates resistant to drugs from all three classes
49 have been reported (12).

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50 Unfortunately, the antifungal drug pipeline is unlikely to yield an abundance of new
51 treatment options in the near future. Antifungal drug development is hampered by intrinsic
52 challenges (fungi, like humans, are eukaryotic organisms, and therefore it is difficult to identify
53 compounds that are active against fungal pathogens but not highly toxic to host cells) and by
54 poor financial incentives for pharmaceutical companies (10). Ibrexafungerp, a first-in-class
55 glucan synthase inhibitor, has recently been shown to have activity against *C. auris* (13) in *in*
56 *vitro* and animal models (14), but has not yet been approved for clinical use.
57 One treatment approach that does not rely on the introduction of novel agents is the
58 repurposing of existing drugs in combination. Combination therapy using two or more
59 antifungal drugs is already an established component of therapy for certain fungal infections:
60 AMB is routinely used in combination with 5-fluorocytosine as induction therapy for
61 *Cryptococcus neoformans* meningitis (15), while combinations of antifungal drugs are often
62 employed in an attempt to treat infections caused by highly drug-resistant molds such as
63 *Scedosporium* spp. (16). This strategy, however, still relies on the limited number of currently
64 available antifungal agents.
65 In 1972, investigators observed *in vitro* synergistic activity in *Saccharomyces cerevisiae*
66 when AMB was combined with rifampin (RIF), an antibacterial RNA synthesis inhibitor (17) or
67 with tetracycline, an antibacterial protein synthesis inhibitor (18); the combination of AMB with
68 RIF was later also noted to be synergistic against several *Candida* species (19). Synergy between
69 AMB and the tetracycline analogue minocycline (MIN) was demonstrated against *Cryptococcus*
70 *neoformans*, *C. albicans*, and other *Candida* species in 1977 (20). Fluconazole was also
71 subsequently reported to be synergistic with MIN and with another tetracycline analogue,

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72 doxycycline, against *C. albicans* (21, 22). To our knowledge, however, these antibacterial-
73 antifungal combinations have not been evaluated in *C. auris*. We used a novel inkjet printer-
74 assisted checkerboard array synergy method as well as time-kill synergy studies to investigate
75 synergistic activity of combinations of MIN, RIF, voriconazole (VRC; an azole antifungal),
76 caspofungin (CAS; an echinocandin), and AMB against a collection of *C. auris* isolates with a
77 range of drug resistance patterns.
78
79 RESULTS
80 **Single drug MIC testing by broth microdilution (BMD) and digital dispensing method (DDM)**.
81 BMD MICs were prepared in triplicate to determine the modal MIC (i.e. the MIC obtained in ≥2
82 replicates) at 24 hours (all drugs) and 48 hours (VRC and AMB) using standard Clinical and
83 Laboratory Standards Institute (CLSI) methodology (23). If three sequential doubling dilution
84 values were obtained in the three replicates (e.g. 2, 4, and 8 μg/mL), the middle value was
85 considered the modal MIC. Modal MICs ranged from 0.5 to 2 μg/mL for AMB, from ≤0.016 to >8
86 μg/mL for VRC, and from 0.063-0.5 μg/mL for CAS. VRC MICs were higher at 48 than 24 hours,
87 while AMB MICs were either the same or one doubling dilution higher at 48 hours compared to
88 24 hours for all strains. Neither RIF nor MIN exhibited inhibitory activity at the concentrations
89 tested (Table 1 and Table S1).
90
91 For each condition (i.e., each drug tested against each strain at 24 or 48 hours), five DDM MIC
92 values were also obtained: a dedicated MIC test result and four results from the single-drug
93 titrations of each synergy grid in the checkerboard array testing described below (Table S1).

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94 When on-scale DDM MIC results were compared to on-scale modal BMD MICs, 94.7% were
95 within ±1 doubling dilution and 99.5% were within ±2 doubling dilutions of the modal MIC
96 (Table 2). In antifungal susceptibility testing, essential agreement between a new method and
97 the reference method is generally defined as an MIC value falling within ±2 doubling dilutions of
98 the reference result (24, 25). Concordance with the modal BMD MIC was highest for AMB
99 (100% of on-scale results within ±1 doubling dilution) and lowest for CAS (81.6% and 98.0% of
100 on-scale results within ±1 and ±2 doubling dilutions, respectively). Therefore, our data indicate
101 that the DDM automation method provides accurate and robust antifungal testing results.
102
103 **Checkerboard array synergy testing using DDM**. Using the checkerboard array assay, we found
104 that MIN was synergistic against all 10 strains when combined with either AMB or CAS, and RIF
105 was synergistic with AMB against 9 strains. In synergistic combinations, the concentration of
106 MIN at the $FIC_{I\text{-}MIN}$ ranged from 4-16 μg/mL and was ≤8 μg/mL in 12 of 22 cases, while the
107 concentration of RIF was 8 μg/mL in 5 cases and 16 μg/mL in 4 cases. All other combinations
108 were synergistic against ≤2 strains. Three of the 5 combinations that were not synergistic
109 against any strains were antagonistic against 2 strains. (Table 3 and Table S2).
110
111 **Time-kill synergy testing**. The three combinations that demonstrated synergy against ≥9 strains
112 by the checkerboard array assay (MIN plus AMB, MIN plus CAS, and RIF plus AMB) were tested
113 using the time-kill method against a subset of 5 of the *C. auris* strains, which were chosen to
114 represent a range of susceptibility profiles. (Table 4, Figure 1). Each drug pair was tested, at two
115 combinations of concentrations of individual drugs, against each of the 5 isolates, and was

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116 considered synergistic and/or fungicidal if at least one of these combinations met criteria for
117 synergistic or fungicidal activity, respectively. At 24 hours, AMB plus RIF and AMB plus MIN
118 were synergistic against all 5 strains, while at 48 hours, AMB plus RIF was synergistic against 4
119 strains and AMB plus MIN against 3 strains. In combination with RIF, AMB showed fungicidal
120 activity against 2 strains at 24 and 48 hours, and against an additional strain at 24 hours, at
121 concentrations at which it was not fungicidal alone. In combination with MIN, AMB showed
122 fungicidal activity against 2 strains at 24 and 48 hours at concentrations at which it was not
123 fungicidal alone. The combination of CAS plus MIN was not synergistic or fungicidal against any
124 strains. The synergy killing curves for this combination were notable in that CAS exhibited an
125 inhibitory or near-inhibitory effect even at concentrations at or below the BMD MIC, yet its
126 effect was unchanged by the addition of MIN. To further investigate this finding, single-drug
127 killing curve studies were performed with CAS over a range of concentrations; these showed
128 minimal effect of drug concentration on inhibition or killing, in accordance with previously
129 reported observations (26) (Figure 2).
130
131 DISCUSSION
132 We identified three combinations of antibacterial and antifungal drugs (AMB plus RIF,
133 AMB plus MIN, and CAS plus MIN) that demonstrated synergistic activity by checkerboard array
134 against ≥90% of *C. auris* strains evaluated; the two AMB-containing combinations were also
135 synergistic by time-kill synergy testing at one or more concentration combinations against the
136 strains evaluated. We thereby demonstrate synergistic activity between antibacterial and
137 antifungal agents against the emerging and highly multidrug-resistant pathogen *C. auris*.

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138 We hypothesize that the mechanism of synergy for these combinations involves
139 impairment in cell wall or membrane integrity by the antifungal drug, permitting entry of an
140 antibacterial agent that would otherwise be unable to access the intracellular compartment of
141 a fungal cell. Echinocandins such as CAS act by inhibiting β-(1,3)-glucan synthase, thus impeding
142 cell wall synthesis and impairing cell wall integrity, resulting in increased vulnerability of the cell
143 to osmotic pressure (27). AMB has traditionally been understood to act by forming ion channels
144 in the lipid bilayer of the fungal cell membrane, thus permeabilizing the cell and ultimately
145 causing cell death (28, 29). However, recent work suggests that large aggregates of AMB, which
146 assemble outside the membrane and act as "sterol sponges" that kill cells by extracting
147 ergosterol from the lipid bilayer, may play a more important role in cell death than do the ion
148 channels (30). It is conceivable that at the subinhibitory concentrations at which it
149 demonstrates synergy with MIN and RIF, AMB could be exerting ion channel permeabilizing
150 activity without aggregate-based cytotoxicity, allowing entry of the antibacterial drugs through
151 the channels.

152 Our hypothesis is supported by prior observations that tetracycline inhibits protein
153 synthesis in isolated yeast ribosomes (31) and that RIF appears to inhibit RNA polymerase in
154 yeast (17). These drugs, therefore, may have targets in yeast cells analogous to those in
155 bacteria but accessible in yeast only in the setting of disruption of cell membrane or cell wall
156 integrity. A similar phenomenon is well established in bacteria, whereby drugs that are unable
157 to bypass the defenses of the Gram-negative outer membrane under normal circumstances
158 demonstrate activity in the presence of low levels of membrane-permeabilizing agents such as
159 polymyxins (32, 33).

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160 We used two separate synergy testing methods in an effort to increase the robustness
161 of our results, and found that while two combinations demonstrated consistent synergy using
162 both methods, a third was synergistic only by the checkerboard array method and not the time-
163 kill method. It is possible that this finding reflects a limitation of the synergistic activity of this
164 combination (CAS plus MIN). Alternatively, the finding may reflect the limitations of the time-
165 kill testing method for evaluation of CAS activity, as our single-drug CAS killing curves did not
166 demonstrate concentration-dependent inhibitory or fungicidal effects, a finding that has been
167 previously observed with this drug in *C. auris* (26).

168 In this study we also demonstrated the utility of an automated inkjet printer-assisted
169 digital dispensing method for MIC and checkerboard array synergy testing in yeast. Manual
170 synergy testing is an error-prone and time-consuming process, and automation allows for
171 significantly higher throughput of the technique, thereby facilitating more rapid investigation of
172 novel combinations. This use of the DDM for MIC and synergy testing of bacteria, first described
173 in our laboratory (34, 35), has been adopted by the United States Centers for Disease Control
174 and Prevention to test drug combinations against multidrug-resistant bacterial pathogens (36)
175 and has the potential for similar use for fungal pathogens such as *C. auris*.

176 *In vitro* synergy testing has certain intrinsic limitations and is not always a direct
177 indicator of *in vivo* efficacy (37). However, identification of combinations with *in vitro* activity
178 provides preliminary data to suggest regimens that may ultimately prove to be of therapeutic
179 benefit. Given the paucity of new antifungal drugs in the development pipeline, regimens that
180 involve readily available drugs for which extensive pharmacokinetic and safety data already
181 exist offer the potential for expedited clinical evaluation and implementation. The

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182 concentrations of antifungal drugs active in the combinations identified were very low and
183 easily clinically achievable. Although no interpretive criteria (i.e. susceptibility breakpoints) exist
184 for MIN or RIF for yeast, the concentration of MIN in synergistic combinations was ≤8 μg/mL in
185 more than half the instances of synergy we identified; such concentrations would be
186 considered susceptible (4 μg/mL) or intermediate (8 μg/mL) for Gram-positive bacterial
187 pathogens such as *Staphylococcus aureus* and *Enterococcus* spp. by CLSI (38), suggesting
188 plausible clinical applicability. In topical or local applications (e.g. ophthalmic drops or catheter
189 coating), antibiotics can often be used at concentrations greater than can be safely achieved
190 systemically (39); the combinations we identified could thus also have potential use in these
191 scenarios.
192 The need for new therapeutic options for *C. auris* has been underscored since the
193 advent of the COVID-19 pandemic, with recent reports from India (4), Colombia (5), and the
194 United States (6) describing *C. auris* infection as a complication of SARS-CoV-2-related critical
195 illness. In addition to possible direct applicability, if the combinations we evaluated act, as we
196 predict, by allowing access of MIN and RIF to intracellular targets in yeast, this information may
197 guide future antifungal drug development approaches. Evaluation of combinations in animal
198 models and ultimately in clinical trials will be critical future steps in establishing clinical activity.
199 In the absence of a predictable timeline for introduction of novel antifungal agents, repurposing
200 existing drugs may be our best hope in identifying new treatment approaches for patients with
201 infections caused by *C. auris* and other emerging multidrug-resistant fungal pathogens.
202
203 MATERIALS AND METHODS

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204 **Fungal isolates**. Ten *C. auris* isolates were obtained from the CDC & FDA Antibiotic Resistance (AR)
205 Isolate Bank (Atlanta, GA). *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, *Escherichia coli*
206 ATCC 25922, and *Staphylococcus aureus* ATCC 29213 were obtained from the American Type Culture
207 Collection (Manassas, VA). All strains were colony purified, minimally passaged, and stored at -80°C in
208 tryptic soy broth (BD Diagnostics, Franklin Lakes, NJ) with 50% glycerol (Sigma-Aldrich, St. Louis, MO).
209

210 **Antimicrobial agents**. Voriconazole (VRC) was obtained from Acros Organics (Pittsburgh, PA).
211 Caspofungin (CAS) was obtained from Carbosynth (Oakbrook Terrace, IL). Amphotericin B (AMB) was
212 obtained from Sigma-Aldrich (St. Louis, MO). Minocycline (MIN) was obtained from Chem Impex
213 International (Wood Dale, IL). Rifampin (RIF) was obtained from Fisher Scientific (Waltham, MA).
214 Antimicrobial stocks were prepared in DMSO (Sigma-Aldrich), with the exception of minocycline stock
215 used for time-kill experiments, which was prepared in water. All antimicrobials were quality control (QC)
216 tested with *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 (VRC, CAS, and AMB) or with *E. coli* ATCC
217 25922 and *S. aureus* ATCC 29213 (MIN and RIF) and were used only if they produced an MIC result in the
218 QC range accepted by CLSI (38, 40). After passing QC, stocks were aliquoted and stored at -80°C
219 (antifungal drugs) or -20°C (antibacterial drugs) until use. Aliquots were discarded after a single use.
220

221 **Antimicrobial susceptibility testing**. Manual broth microdilution (BMD) testing of *C. auris* isolates was
222 performed in triplicate for each drug according to CLSI guidelines (23). Strains were isolation streaked on
223 Sabouraud dextrose agar plates (Thermo Scientific, Waltham, MA) and incubated for 24 hours at 35°C in
224 ambient air. BMD plates were made by preparing serial 2-fold dilutions of antimicrobial agents at twice
225 the desired final concentration in 100 μL RPMI 1640 media with L-glutamine (Cytiva, Marlborough, MA)
226 prepared with MOPS buffer (Fisher Scientific, Waltham, MA) in clear, round-bottom, untreated 96-well
227 plates (Evergreen Scientific, Los Angeles, CA, USA). Fungal inocula were prepared by suspending colonies

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228 from the overnight plates in sterile 0.9% sodium chloride and adjusting to a 0.5 McFarland standard,
229 diluting this suspension 1:1000 in RPMI, then adding 100 μL of the diluted suspension to each well for a
230 final volume of 200 μL and cell density of 0.5-2.5 x10^3 CFU/mL. Negative (sterility) control and growth
231 control wells were included in each row. Plates were then incubated at 35°C in ambient air. At 24 hours
232 (all drugs) and 48 hours (VRC and AMB), plates were removed from the incubator and vortexed on a
233 plate shaker for 4 minutes, after which OD_{600} readings were taken with a Tecan Infinite M1000 Pro
234 microplate reader (Tecan, Morrisville, NC) to quantify growth. OD_{600} readings were normalized by
235 subtracting the average reading of the negative control wells from the same plate, which contained
236 media without yeast, then the percent inhibition for each well was calculated relative to the average of
237 the positive growth control wells of the same isolate from the same plate. For CAS and VRC, the lowest
238 concentration of drug that reduced growth by at least 50% was considered the MIC; for AMB the lowest
239 concentration of drug that reduced growth by at least 90% was considered the MIC (23). If a skipped
240 well occurred, MIC testing was repeated.
241
242 MIC testing was also performed using an automated inkjet printer-assisted digital dispensing method
243 (DDM) adapted from a method developed in our laboratory for MIC testing of bacteria (34). Initial 0.5
244 McFarland yeast suspensions were prepared in RPMI as described above and then diluted 1:2000 in
245 RPMI. The same final volume and cell density in each well was achieved by adding 200 μL of this diluted
246 suspension to each well in a 96-well plate. Antimicrobial drugs were then dispensed by the HP D300
247 digital dispenser instrument (HP, Inc., Palo Alto, CA) into the yeast suspension in the wells. Incubation
248 and growth interpretation were carried out as in the BMD method described above.
249
250 **Checkerboard array synergy testing**. The DDM method described above was used to prepare
251 checkerboard arrays in which two drugs were each dispensed in 7-9 two-fold dilutions. Each

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252 combination was tested against every *C. auris* strain, with growth determinations made after 24 hours
253 of incubation. Growth inhibition was determined as described above. For combinations in which both
254 drugs use 90% inhibition for MIC determination (AMB plus MIN, AMB plus RIF, and MIN plus RIF),
255 combination wells were considered inhibitory when growth was inhibited by 90% relative to growth
256 control wells; for all other combinations, combination wells were considered inhibitory when growth
257 was inhibited by at least 50% relative to growth control wells. For each combination well in which
258 growth was inhibited, the fractional inhibitory concentration (FIC) for each drug was calculated by
259 dividing the concentration of the drug in that well by the MIC of that drug alone. The FIC index (FIC_I) for
260 the well was then calculated by summing the FICs of the two drugs in the well; in cases where the MIC of
261 a drug was off-scale, the highest concentration tested was assigned an FIC of 0.5 to permit calculation of
262 the FIC_I. A combination was considered synergistic against an isolate if it had a minimum FIC_I (FIC_{I-MIN}) of
263 ≤0.5, antagonistic if it had an FIC_{I-MIN} of >4.0, and indifferent if it had an intermediate FIC_{I-MIN} value (41).
264

265 **Time-kill synergy testing**.

266 Antimicrobial stocks were diluted in 9.5 mL of RPMI 1640 in 25- by 150-mm glass round-bottom tubes to
267 the appropriate starting concentrations, which were selected based on checkerboard array synergy
268 results. For the AMB-containing combinations, two different AMB concentrations (chosen based on
269 checkerboard array results) were tested in combination with a fixed concentration of MIN or RIF. For the
270 combination of CAS plus MIN, two concentrations of MIN were tested with a fixed concentration of CAS
271 because of the observation that the effect of CAS on yeast growth was minimally affected by CAS
272 concentration (see results and discussion). Negative (sterility) control and positive growth control tubes
273 containing no antimicrobials were also prepared. A 1.0 McFarland suspension of yeast cells from an
274 overnight plate was prepared in 0.9% sodium chloride and 0.5 mL of this suspension was added to each
275 tube for a final starting concentration of 1-5x10^5 CFU/mL. Cultures were incubated with shaking in

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276 ambient air at 35°C for 48 hours. At 0, 3, 6, 24, and 48 hours, aliquots were removed from the culture
277 tube and a 10-fold dilution series was prepared in 0.9% sodium chloride. A 10 µL drop from each dilution
278 was plated onto Sabouraud dextrose agar and incubated overnight. The colonies within each drop were
279 then counted; drops containing 3 to 40 colonies were considered usable and cell density was calculated
280 from these. If more than one dilution for a given sample was usable, the cell densities of the two drops
281 were averaged. If no drops were usable, the densities for consecutive drops above and below the usable
282 range were averaged. The lower limit of detection with this method is 300 CFU/mL. A combination was
283 considered synergistic if it resulted in a ≥2 $\log_{10}$ reduction in CFU/mL compared to the most active agent
284 alone and fungicidal if it resulted in a ≥3 $\log_{10}$ CFU/mL reduction compared to starting inoculum. Synergy
285 and fungicidal activity were evaluated at 24 and 48 hours.

286

287 **Data analysis**. Data output from plate readings was visualized using Microsoft Excel (Microsoft
288 Corporation, Redmond, WA). A custom Python script was used to normalize MIC and synergy results and
289 to calculate and visualize growth inhibition.

290

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412 FIGURE LEGENDS

413 FIG 1 Time-kill synergy graphs

414 Strain numbers refer to CDC & FDA Antibiotic Resistance Isolate Bank designations. Dashed line indicates

415 assay lower limit of detection. Filled (red) symbols indicate synergistic concentration combinations.

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417 FIG 2 Caspofungin killing curves

418 Strain numbers refer to CDC & FDA Antibiotic Resistance Isolate Bank designations. The broth

419 microdilution MIC of caspofungin is 0.5 µg/mL for strain 0388 and 0.13 µg/mL for strain 0389.

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421 TABLES

TABLE 1 Modal broth microdilution MICs (µg/mL) at 24 and 48 hours

Strain (AR Bank Number)	AMB 24 hr	AMB 48 hr	VRC 24 hr	VRC 48 hr	CAS 24 hr	MIN 24 hr	RIF 24 hr
0381	0.5	1	≤0.016	0.031	0.063	>64	>128
0382	0.5	1	0.031	>8	0.125	>64	>128
0383	1	1	1	2	0.125	>64	>128
0384	1	1	0.5	N/A	0.125	>64	>128
0385	1	1	8	>8	0.125	>64	>128
0386	1	1	4	>8	0.125	>64	>128
0387	1	1	0.063	>8	0.125	>64	>128
0388	2	2	1	4	0.5	>64	>128
0389	2	2	4	8	0.125	>64	>128
0390	1	2	1	2	0.125	>64	>128

AR Bank: CDC & FDA Antibiotic Resistance Isolate Bank; AMB, amphotericin B; CAS, caspofungin; VRC, voriconazole; MIN, minocycline; RIF, rifampin. N/A: Modal MIC could not be determined as MICs were 0.125, 2, and 8 µg/mL

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TABLE 2 Essential agreement between on-scale DDM MIC results and modal BMD MIC

Drug and time	DDM MIC results within **±1** two-fold dilution of modal MIC (*n*, %)	DDM MIC results within **±2** two-fold dilutions of modal MIC (*n*, %)
AMB, 24 hr	50/50 (100.0)	50/50 (100.0)
AMB, 48 hr	49/49 (100.0)	49/49 (100.0)
CAS, 24 hr	40/49 (81.6)	48/49 (98.0)
VRC, 24 hr	35/37 (94.6)	37/37 (100.0)
VRC, 48 hr	21/21 (100.0)	21/21 (100.0)
MIN, 24 hr	N/A	N/A
RIF, 24 hr	N/A	N/A
Total	195/206 (94.7)	205/206 (99.5)

AMB, amphotericin B; CAS, caspofungin; VRC, voriconazole; MIN, minocycline; RIF, rifampin.

MIC results were not used when >1 skipped well occurred (n = 6), when modal BMD MIC or DDM result was off-scale (n = 33), or when there was no modal BMD MIC (n = 5).

N/A: Essential agreement could not be calculated due to off-scale high modal BMD MICs; all DDM results were also off-scale high for these drugs.

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TABLE 3 Checkerboard array synergy results

Drug Combination	Percent of strains against which combination was **synergistic** (*n* = 10)	Percent of strains against which combination was **antagonistic** (*n* = 10)
AMB + MIN	100%	0%
CAS + MIN	100%	0%
AMB + RIF	90%	0%
MIN + VRC	20%	0%
CAS + VRC	10%	0%
MIN + RIF	0%	0%
AMB + CAS	0%	0%
AMB + VRC	0%	20%
CAS + RIF	0%	20%
RIF + VRC	0%	20%

AMB, amphotericin B; CAS, caspofungin; VRC, voriconazole; MIN, minocycline; RIF, rifampin.

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TABLE 4 Time-kill results

Strain (AR Bank Number)	Drug concentrations (µg/mL)	Concentration difference of cells treated with combination vs most active single agent (log_{10} CFU/mL) **Shaded cells indicate *synergy***		Concentration difference at end vs start of cells treated with combination (log_{10} CFU/mL) **Shaded cells indicate *fungicidal activity***	
		24 hours	48 hours	24 hours	48 hours
0381	AMB 0.125 + RIF 4	-4.14	-2.17	-2.94	-0.61
	AMB 0.25 + RIF 4	-2.03	-4.30	-3.12	-3.12
0382	AMB 0.25 + RIF 4	-2.37	-4.78	-3.21	-3.21
	AMB 0.5 + RIF 4	0.00	-2.85	-2.70	-2.70
0383	AMB 0.125 + RIF 8	-1.80	-0.50	0.44	1.74
	AMB 0.25 + RIF 8	-3.98	-3.83	-2.39	-1.99
0388	AMB 0.25 + RIF 16	-0.40	0.07	1.57	1.91
	AMB 0.5 + RIF 16	-4.82	-0.82	-2.78	1.05
0389	AMB 0.5 + RIF 16	-3.04	-0.80	-1.38	1.00
	AMB 1 + RIF 16	-2.43	-1.51	-3.52	-0.30
0381	AMB 0.125 + MIN 8	-2.30	-0.60	-1.15	0.95
	AMB 0.25 + MIN 8	-2.08	-4.78	-3.19	-3.19
0382	AMB 0.125 + MIN 8	-2.34	-0.75	-0.80	1.41
	AMB 0.25 + MIN 8	-1.30	-2.76	-1.64	-1.16
0383	AMB 0.125 + MIN 8	-2.02	-0.01	0.13	2.02
	AMB 0.25 + MIN 8	-3.12	-2.34	-1.30	-0.60
0388	AMB 0.5 + MIN 8	-2.37	-0.06	-0.60	1.74

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	AMB 1 + MIN 8	0.62	-1.30	-0.64	-0.43
0389	AMB 0.5 + MIN 16	-0.53	0.11	-0.96	1.77
	AMB 1 + MIN 16	-3.48	-4.99	-3.30	-3.30
0381	CAS 0.031 + MIN 8	-0.34	-0.34	-0.02	-0.18
	CAS 0.031 + MIN 16	-0.46	0.00	-2.46	-2.00
0382	CAS 0.125 + MIN 8	1.07	0.84	-0.27	-0.36
	CAS 0.125 + MIN 16	1.02	0.96	-1.24	-1.78
0383	CAS 0.063 + MIN 4	0.65	1.26	0.20	0.34
	CAS 0.063 + MIN 8	0.63	1.00	-1.81	-1.57
0388	CAS 0.125 + MIN 8	0.35	0.85	-0.18	0.44
	CAS 0.125 + MIN 16	0.27	0.39	-1.66	-1.55
0389	CAS 0.25 + MIN 1	0.77	0.65	0.37	0.20
	CAS 0.25 + MIN 2	0.38	0.78	-1.72	-1.46

AR Bank: CDC & FDA Antibiotic Resistance Isolate Bank; AMB, amphotericin B; CAS, caspofungin; MIN, minocycline; RIF, rifampin.

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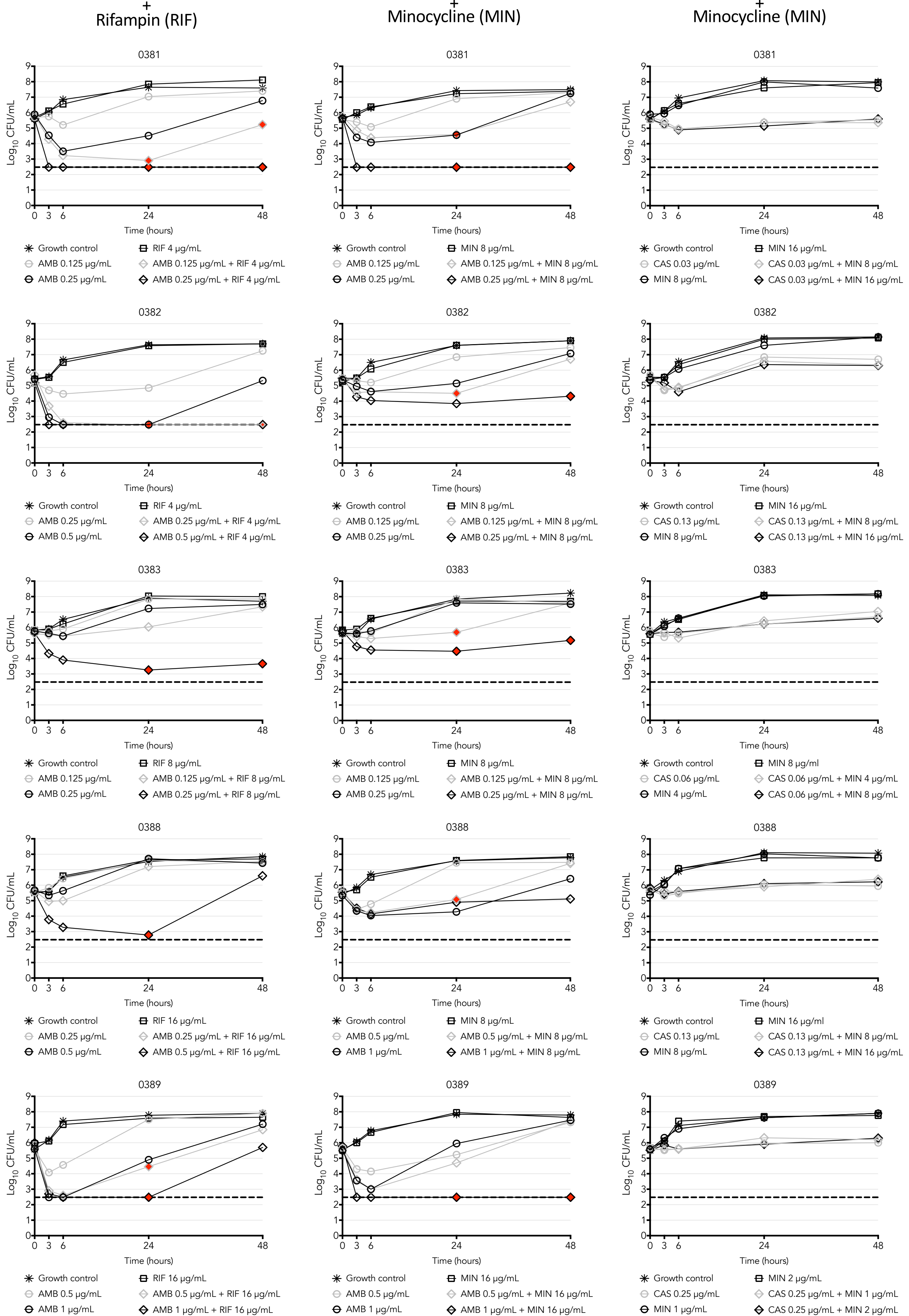

FIGURE 1 Time-kill synergy graphs

Strain numbers refer to CDC & FDA Antibiotic Resistance Isolate Bank designations. Dashed line indicates assay lower limit of detection. Filled (red) symbols indicate synergistic concentration combinations.

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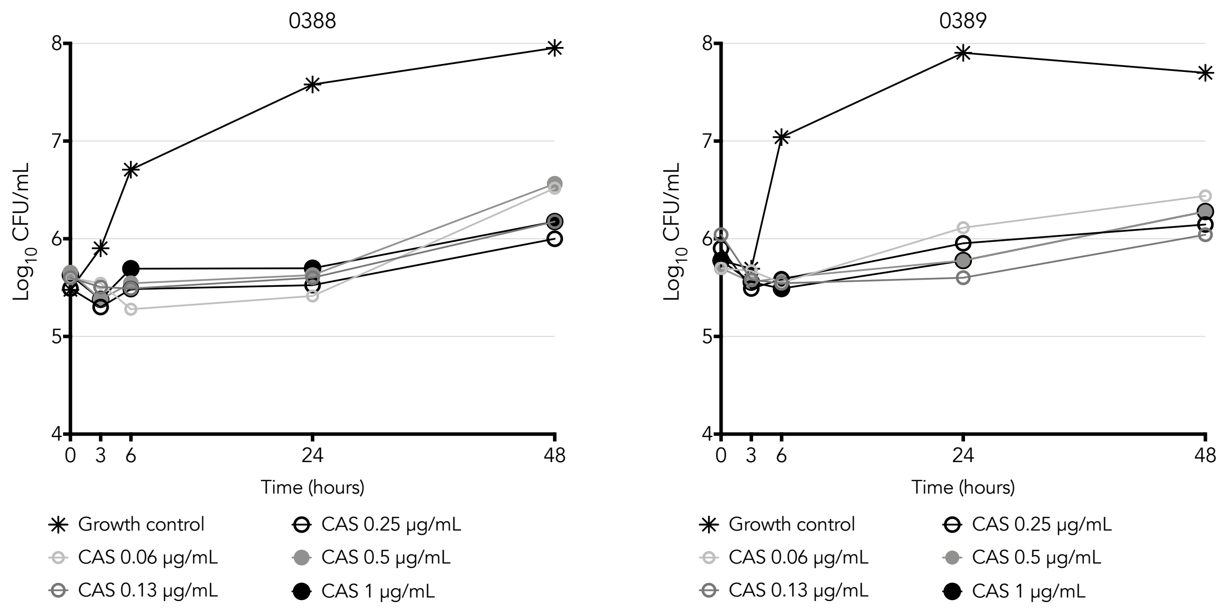

FIGURE 2 Caspofungin killing curves

Strain numbers refer to CDC & FDA Antibiotic Resistance Isolate Bank designations. The broth microdilution MIC of caspofungin is 0.5 µg/mL for strain 0388 and 0.13 µg/mL for strain 0389.