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SUSCEPTIBILITY

Screening a Repurposing Library for Inhibitors of Multidrug-Resistant *Candida auris* Identifies Ebselen as a Repositionable Candidate for Antifungal Drug Development

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ABSTRACT Since its original isolation in 2009, *Candida auris* has spread across the globe as a causative agent of invasive candidiasis. *C. auris* is typically intrinsically resistant to fluconazole and can also be resistant to echinocandins and even amphotericin B. Thus, there is an urgent need to find new treatment options against this emerging pathogen. To address this growing problem, we performed a screen of the Prestwick Chemical library, a repurposing library of 1,280 small molecules, consisting mostly of approved off-patent drugs, in search of those with activity against a multidrug-resistant *C. auris* isolate. Our initial screen, using standardized susceptibility testing methodologies, identified nine miscellaneous compounds with no previous clinical indication as antifungals or antiseptics that displayed activity against *C. auris*. Confirmation and follow-up studies identified ebselen as the drug displaying the most potent activity, with 100% inhibition of growth detected at concentrations as low as 2.5 μM. We further evaluated the ability of ebselen to inhibit *C. auris* biofilm formation and examined the effects of combination therapies of ebselen with clinically used antifungals. We extended our studies to different *C. auris* strains with various susceptibility patterns and also confirmed its antifungal activity against *Candida albicans* and clinical isolates of multiple other *Candida* species. Furthermore, ebselen displayed a broad spectrum of antifungal actions on the basis of its activity against a variety of medically important fungi, including yeasts and molds. Overall, our results indicate the promise of ebselen as a repositionable agent for the treatment of candidiasis and possibly other mycoses and, in particular, for the treatment of infections refractory to conventional treatment with current antifungals.

KEYWORDS *Candida auris*, antifungals, Prestwick library, repurposing, candidiasis

Candidiasis represents a significant challenge to clinicians, as *Candida* species are currently the third to fourth most common cause of nosocomial bloodstream infections in hospitalized patients (1–3). Invasive candidiasis carries substantial morbidity and mortality, with crude mortality rates approaching 40% (4). Although *Candida albicans* remains the main causative agent of this infectious disease, non-*albicans Candida* spp. account for approximately half the cases. Most recently, attention has focused on the emerging pathogen *Candida auris*. First described in 2009 as an isolate collected from the external ear canal of a patient in Japan (5), *C. auris* has quickly spread and become a growing threat in hospitals throughout Asia, Europe, South America, and

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more recently, the United States (6–10). Unlike *C. albicans*, this emerging pathogen has the ability to live on surfaces outside the human body, further complicating the management of these infections by health care facilities (11, 12). For example, an outbreak of *C. auris* infections in London from April 2015 to July 2016 had 50 cases, with 44% of patients developing candidemia, and strict infection and prevention control measures had to be implemented to slow hospital transmission (12, 13). In a recent retrospective review of the clinical history of 54 patients, most had multiple risk factors for invasive disease, and candidemia was observed in 61% (8). Strikingly, the mortality rate in this series of patients was 59%. In the United States, as of 31 December 2017, the number of documented cases of infection caused by this species has escalated to a total of 228 (14), which is markedly higher than just a few months before (15, 16). Unfortunately, antifungal therapy against invasive infections caused by this emerging pathogen may be limited, as up to 90% of isolates are resistant to fluconazole and 50% have reduced susceptibility to other azoles. Currently, the echinocandins are recommended for the treatment of *C. auris* infections, but elevated MICs secondary to hot spot regions in the *FKS* genes (*FKS1* and *FKS2*) known to cause resistance to the echinocandins in other *Candida* species and *Aspergillus fumigatus* have been found in a proportion of *C. auris* isolates (17, 18). A few isolates have been documented to be resistant to all clinically available classes of antifungal agents, including amphotericin B. In addition, although *C. auris* does not form hyphae, it has the ability to form biofilms which can also contribute to high levels of resistance (19). To make matters worse, this species is also often misidentified by commercially available automated systems that use biochemical means for species identification (20, 21). This may further negatively affect outcomes if inappropriate therapy is initiated on the basis of an incorrect species identification.

Repurposing or repositioning existing drugs can considerably decrease the effort, time, and expense that it takes to develop novel antifungals (22), which represents a pressing and unmet clinical need, as highlighted by the emergence of multidrug-resistant *C. auris*, its spread, and the increasing number of infections caused by this recently described pathogen (23, 24). Drug repurposing consists of the evaluation of drugs that are already approved or undergoing evaluation for the treatment of other diseases and, for the purpose of this study, have no previous clinical indications as antifungals (25, 26). To this extent, we screened a repurposing library in search of drugs with antifungal activity against a multidrug-resistant *C. auris* isolate. The Prestwick Chemical library consists mostly of an FDA-approved off-patent collection of approximately 1,200 small molecules (80 to 1,670 g/mol) with a wide range of functions and mechanisms of action that are used as drugs for a variety of diseases, including infectious, neurodegenerative, psychiatric, and cardiovascular diseases and cancer. Moreover, since the structure, chemical properties, and biological functions of almost all members of this library are known, we would expect any hits obtained in our antifungal screen to be easier to interpret and also facilitate the further analysis of the novel functionality of the established molecule (27). Our results identified ebselen, an organoselenium compound currently undergoing clinical trials for the treatment of different diseases (28, 29), as the leading repositionable candidate with potent antifungal activity against *C. auris*.

RESULTS AND DISCUSSION

Screening the Prestwick library for inhibitors of *C. auris* growth. We performed a primary screen of the Prestwick Chemical library, at a 20 μM fixed concentration, to identify inhibitors of *C. auris* growth, for which we used a 96-well microtiter plate-based model mostly in accordance with Clinical and Laboratory Standards Institute (CLSI) methodologies with slight modifications. This initial screen was conducted using *C. auris* 0390 strain from the Centers for Disease Control and Prevention (CDC) panel (30), which is resistant to fluconazole and amphotericin B and also has decreased susceptibility against echinocandins. The screen was conducted in duplicates (using independent plates), and the results are expressed as a percentage inhibition of growth

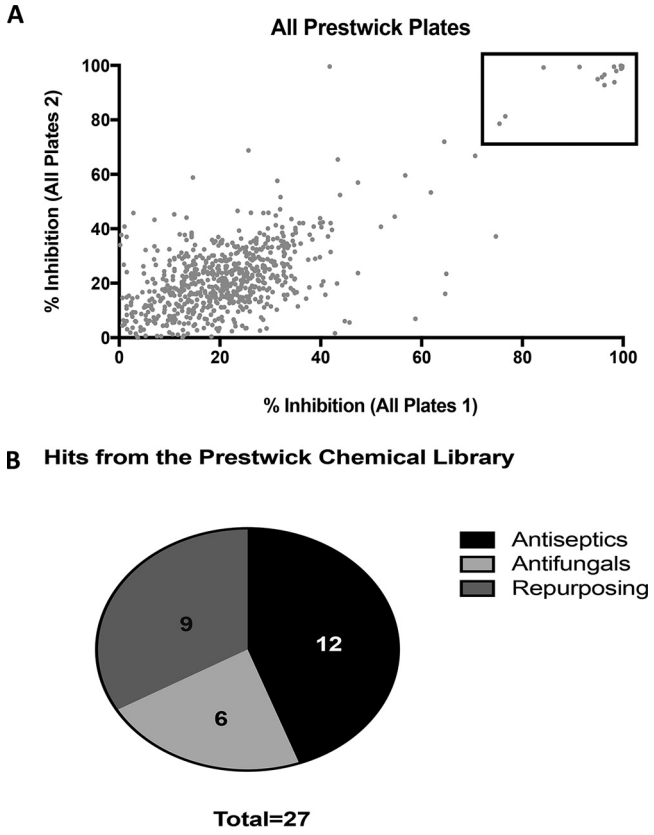

FIG 1 Primary screening of the Prestwick Chemical library. (A) Graphical representation of results of primary screening. The experiment was performed in duplicates and results expressed as percent inhibition according to OD_{490} spectrophotometric readings. (B) Initial hits were classified into three classes: antiseptics, antifungals, and repositionable agents.

compared to that of untreated controls, estimated from spectrophotometric readings at the end of the 48-h incubation period (Fig. 1A and Table 1). On the basis of this primary screen, we identified 27 initial hit compounds that inhibited growth by >70% (Fig. 1A), resulting in a 2.1% initial hit rate. These 27 hits were initially classified into three major categories, including 12 antiseptics/antimicrobials, 6 known antifungal agents, and 9 other miscellaneous drugs with no previous clinical indication as antifungals (Fig. 1B and Table 1; see also Table S1 in the supplemental material). Because the main emphasis of this work was to find drugs that can be repurposed as antifungals for the treatment of invasive infections, follow-up experiments were focused on these miscellaneous drugs. However, we note that since, unlike *C. albicans*, *C. auris* has the

TABLE 1 Potential repositionable hit compounds identified during the primary screen of the Prestwick library and their corresponding IC_{50}s against *C. auris* strain 0390 planktonic growth calculated from dose-dependent confirmatory experiments

Drug Name	Drug type	% inhibition (from primary screen)	IC_{50} (μM)
R(-)-Apomorphine hydrochloride hemihydrate	Emetic	>90	6.928
Suloctidil	Vasodilator	>90	Not confirmed
Ebselen	Anti-inflammatory	>90	1.413
Nisoldipine	Antihypertensive	79	29.42
Argatroban	Anticoagulant	>90 (on single plate)	Not confirmed
Dimethisoquin hydrochloride	Local anesthetic	70	28.61
Pentetic acid	Chelating agent/radioactive decontaminant	>90	11.15
Pentamidine isethionate	Antiparasitic	87	72.94
Pyrvinium pamoate	Antiparasitic	75	7.192

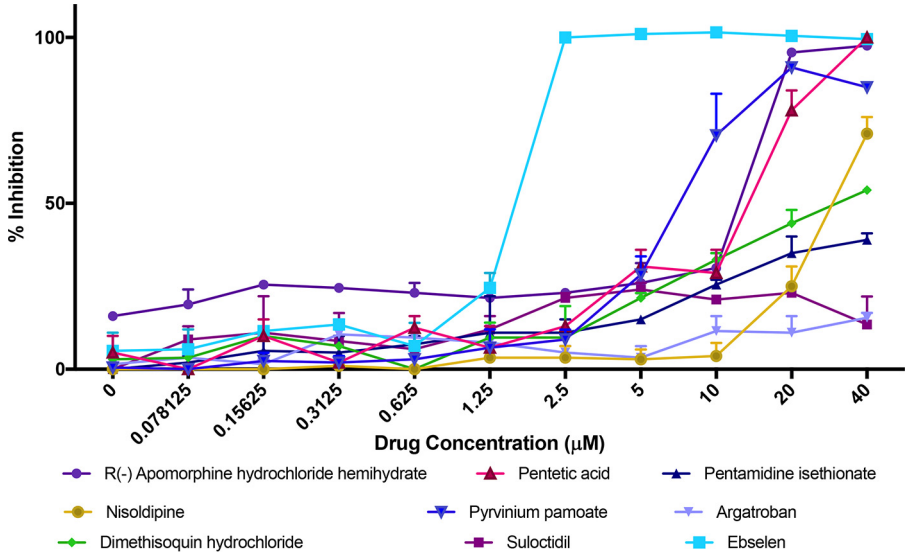

FIG 2 Dose-response assays to confirm the inhibitory activity and determine the potency of the repositionable compounds identified as initial hits in the primary screen. Experiments were performed in two independent plates with two duplicate wells per plate. Bars indicate standard errors.

ability to live on surfaces outside the human body, the identification of different antiseptics (Table S1) with activity against this emerging nosocomial pathogen can also be important for the disinfection of surfaces that could help curtail microepidemics in health care facilities (12, 31, 32). Regarding the established antifungals (Table S1), as expected and confirming the validity of our screening technique, the results of the primary screen confirmed the lack of activity of both fluconazole and amphotericin B (echinocandins are not represented in the Prestwick library). As expected, most other azole derivatives lacked activity in this screen. A few clinically available antifungals, including voriconazole, were represented among the hits, possibly due to the relatively high concentration at which we performed the initial screen. *C. auris* strain 0390 is reported to be resistant to 5-flucytosine (30), and so it was somewhat surprising to see this antifungal in the initial list of hits for this primary screen. Another antifungal compound with activity in this screen, ciclopirox ethanolamine, is used for the topical dermatologic treatment of mycoses and could represent an option for the treatment of superficial infections caused by *C. auris* (33).

Dose-response assays to confirm the activity and establish the potency of initial hit compounds. The nine miscellaneous drugs with no previous clinical indication as antifungals identified as hits in the primary screen were apomorphine, suloctidil, ebselen, nisoldipine, argatroban, dimethisoquin, pentetic acid, pentamidine isethionate, and pyrvinium pamoate. These compounds have a variety of original classifications as emetic, antihypertensive, antiparasitic, and anti-inflammatory drugs (Table 1). To confirm their inhibitory effects on *C. auris* growth, we performed dose-response assays using the same method used for the primary screen but tested their inhibitory activity over a range of drug concentrations, from 40 to 0.078 μM (Fig. 2). From the resulting dose-response curves, we calculated the 50% inhibitory concentration (IC_{50}) for each known drug, a measure of the antifungal potency of each compound against *C. auris* (Table 1). Although we confirmed the activity of a majority of these initial hit compounds, from these experiments, ebselen clearly emerged as the top drug with potent inhibitory activity against *C. auris*, with concentrations as low as 2.5 μM completely abrogating the growth of this multidrug-resistant strain (Fig. 2). Notably, this concentration is well within the physiologically achievable levels of ebselen according to previous studies (34, 35). Thus, we decided to focus our attention on this drug.

Ebselen [2-phenyl-1,2-benzisoselenazol-3(2H)-one] (see Fig. S1) is a synthetic organoselenium compound that is part of the NIH clinical collection, and despite exten-

sive research, its target molecules and mechanism of action are not completely understood (36–40). Due to its highly electrophilic nature, it interacts with cysteine-rich proteins (including thioredoxin) as well as with nonproteins such as thiols (41, 42). It is generally considered an antioxidant, mimicking glutathione peroxidase activity and catalyzing the reduction of reactive oxidase species (25, 29, 43), leading to the attenuation of damage caused by oxidants and radicals. Although it is not currently FDA approved, it is considered clinically safe and is undergoing clinical trials for different indications, including stroke, arthritis, hearing loss, cardiovascular disease, atherosclerosis, and cancer (28, 44). In addition, the antibacterial activity of ebselen has been demonstrated, which may be related to the inhibition of protein synthesis (45–47). Interestingly, the activity of ebselen against *Saccharomyces cerevisiae* was recently described, which was associated with the induction of reactive oxygen species (ROS)-mediated cytotoxicity and the membrane H^+-ATPase pump (Pma1p) in yeast, already hinting to its potential antifungal activity (48). These observations were subsequently confirmed in elegant studies by the Seleem group, demonstrating the antifungal activity of ebselen against pathogenic yeast species by regulating glutathione and ROS production in fungal cells and depleting intracellular glutathione levels (41). Altogether, these data confirm our observations and provide further credence to the potential of ebselen to be repurposed as an antifungal drug.

Activity of ebselen against several *C. auris* clinical isolates from the CDC panel. We expanded our experiments to examine the ability of ebselen to inhibit planktonic growth of all 10 *C. auris* clinical isolates present in the CDC panel (30). For these experiments, we used the same 96-well microtiter plate method as for the dose-response assays described above, a slightly modified version of CLSI susceptibility techniques, with ebselen concentrations ranging from 32 to 0.0625 μg/ml. From the resulting dose-response assays (Fig. 3), we calculated the corresponding IC_{50}s of ebselen against the different *C. auris* strains, which ranged from 0.2345 to 1.47 μg/ml (see Table S2). The overall results indicated the potent activity of ebselen against all 10 *C. auris* strains tested, irrespective of their patterns of susceptibility to current antifungals (see also boxes in Fig. 3 for specific information on MIC values for fluconazole, amphotericin B, and caspofungin against each *C. auris* strain, previously determined at the CDC).

Activity of ebselen against *C. auris* biofilms. *Candida* spp. display the ability to form biofilms on inert and biological surfaces, and biofilm formation further complicates treatment due to the high levels of resistance against most clinically used antifungals (49). For example, *Candida* cells within biofilms are up to 1,000 times more resistant to fluconazole than their planktonic counterparts (50). *C. auris* is not an exception to this rule; although a poorer biofilm former than *C. albicans*, its ability to form biofilms has recently been described (19, 51). Particularly worrisome are the facts that the ability of *C. auris* to form biofilms has been associated with increased virulence and poorer patient outcomes and that *C. auris* biofilms are intrinsically resistant to all clinically used antifungals, including the echinocandins (19). Thus, there is an urgent need for new antifungals with activity against *C. auris* biofilms. To examine the ability of ebselen to inhibit *C. auris* biofilm formation, all 10 *C. auris* clinical isolates in the CDC panel were grown in flat-bottomed 96-well polystyrene microtiter plates in the presence or absence of different concentrations of the drug. The efficacy of ebselen to inhibit biofilm formation was assessed by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay that measures the metabolic activity of sessile cells within the biofilms. From the corresponding dose-response assays (Fig. 4), we determined the IC_{50}s at which ebselen inhibits biofilm formation by the different *C. auris* strains tested, as shown in Table S2. The results indicate a reasonable level of activity of ebselen against *C. auris* biofilms, with biofilm IC_{50}s ranging from 5.864 to 9.781 μg/ml, as the drug is able to inhibit biofilm formation at concentrations that are only slightly elevated compared to those that inhibit planktonic *C. auris* populations and are still within the achievable clinical levels (34, 35). In parallel experiments, we also determined the ability of established antifungals to inhibit the

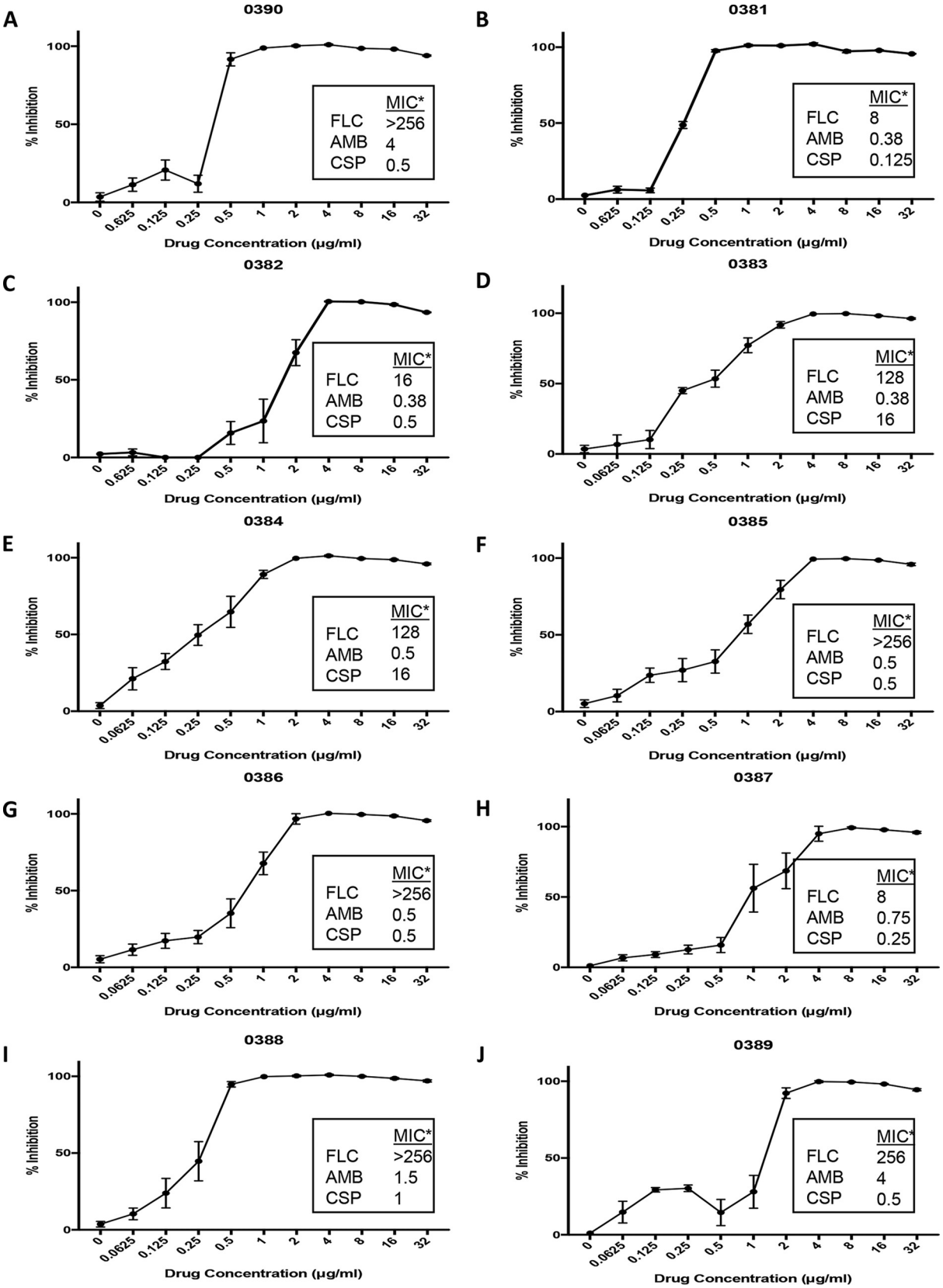

FIG 3 Inhibition of planktonic growth of 10 *C. auris* strains by ebselen. Graphs depict dose-response activity of ebselen against the different *C. auris* strains from the CDC panel. The corresponding MIC values for fluconazole, amphotericin B, and caspofungin are included for comparison purposes (*, provided by the CDC, determined by Etest). Experiments were performed in two independent plates with two duplicate wells per plate. Bars indicate standard errors.

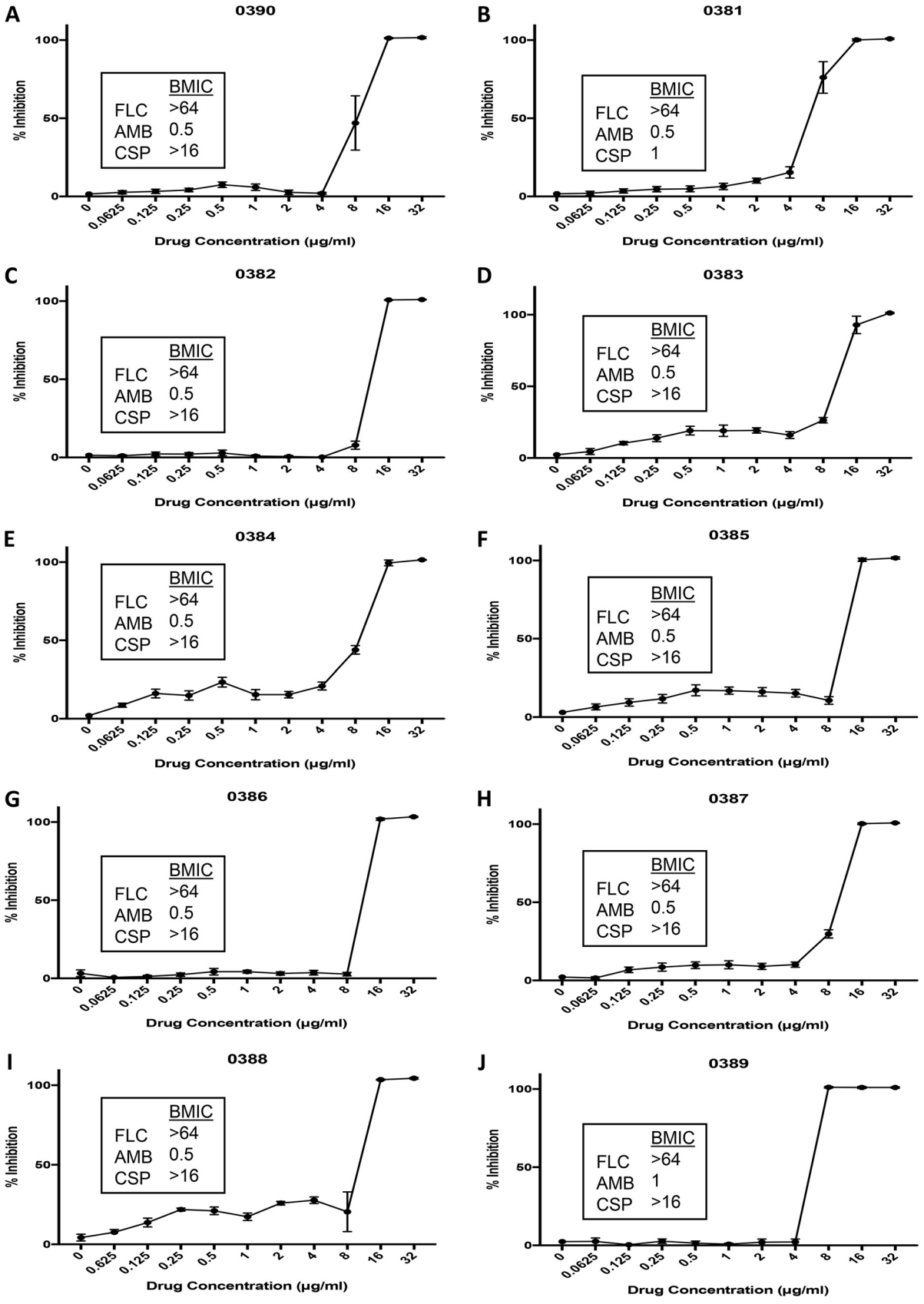

FIG 4 Inhibition of biofilm formation of 10 *C. auris* strains by ebselen. Graphs depict dose-response biofilm-inhibitory activity of ebselen against the different *C. auris* strains from the CDC panel. The corresponding BMIC values, established at 80% inhibition, for fluconazole,

(Continued on next page)

TABLE 2 Calculated IC_{50}s of ebselen for inhibition of planktonic growth and inhibition of biofilm formation for representative strains of other *Candida* species

Candida species	IC_{50} (μg/ml [95% CI[a]])	
	Planktonic	Biofilm
C. albicans	2.832 (2.634–3.044)	8.967 (≤9.514)
C. dubliniensis	0.4012 (0.3728–0.431)	5.01 (3.514–7.282)
C. tropicalis	0.6979 (0.5217–0.9181)	10.35 (≤11.4)
C. parapsilosis	0.9341 (0.8296–1.047)	4.296 (3.106–5.891)
C. glabrata	0.537 (undetermined)	4.705 (undetermined)
C. lusitaniae	0.2913 (≤0.3043)	6.867 (undetermined)
C. krusei	1.421 (1.298–1.548)	2.843 (2.419–3.325)

[a]CI, confidence interval.

formation of *C. auris* biofilms (see boxes in Fig. 4 showing biofilm MIC [BMIC] values for fluconazole, amphotericin B, and caspofungin against each *C. auris* isolate tested). The results indicated that amphotericin B was moderately effective; however, both fluconazole and caspofungin were completely ineffective at inhibiting the biofilm formation of all different *C. auris* isolates tested. This is in agreement with a previous report demonstrating that, contrary to those of *C. albicans*, *C. auris* biofilms are intrinsically resistant to echinocandins (19). Thus, the biofilm inhibitory activity of ebselen compares favorably to those obtained with current antifungal drugs, in particular, fluconazole and caspofungin. Figure S2 shows the crystal violet staining of biofilms formed by all 10 strains of *C. auris*, as well as the dose-response curve for ebselen in this assay. Microscopy was performed for the control biofilms of each strain to show their biofilm-forming capability, while the dose-response curves showed the ability of ebselen to inhibit biofilm formation through the estimation of biofilm biomass compared to that of the untreated control. The results of this assay indicated that, regardless of biofilm-forming ability, ebselen demonstrated inhibitory activity against all *C. auris* isolates tested, thereby confirming our observations from the XTT colorimetric assay.

Activity of ebselen against different *Candida* species. Having demonstrated the activity of ebselen against *C. auris*, we were interested in determining its spectrum of activities against other *Candida* species. For these experiments, we used representative isolates of different *Candida* species, including *C. lusitaniae* and *C. krusei* strains from the same CDC panel (30), the laboratory strain *C. albicans* SC5314, and clinical isolates of *C. dubliniensis*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* from the Fungus Testing Laboratory collection. Susceptibility testing was performed both under planktonic and biofilm growing conditions using the same 96-well microtiter plate methods described above. Table 2 shows the calculated IC_{50}s for the different *Candida* spp. for the inhibition of both planktonic and biofilm growth, while the corresponding dose-response graphs are shown in Fig. S3 and S4. Although some differences between the species were observed, the results indicate that ebselen is active against all *Candida* spp. tested, with planktonic IC_{50}s generally in the range of 0.5 to 2 μg/ml and biofilm IC_{50}s only slightly higher, in the range of 2 to 8 μg/ml.

Activity of ebselen in combination with clinically used antifungal drugs. A very likely scenario in the clinics is that repurposed drugs, such as ebselen, may be used in conjunction with clinically used and currently approved antifungals (52). Thus, we performed experiments to determine the activity of ebselen in combination with conventional antifungal agents. We tested for synergistic, indifferent, or antagonistic effects of the combinations of ebselen with fluconazole, amphotericin B, and caspofungin against *C. auris* and *C. albicans* under two different conditions, including the inhibition of planktonic growth and the inhibition of biofilm formation. As seen in Tables S3 and S4, we observed indifference in the overwhelming majority of paired

FIG 4 Legend (Continued)
amphotericin B, and caspofungin against each strain were also determined and are included in the boxes. Experiments were performed in two independent plates with two duplicate wells per plate. Bars indicate standard errors.

TABLE 3 MIC values of ebselen and current antifungals against multiple clinical isolates belonging to different fungal species for determination of antifungal action

	MIC (μg/ml)					
	50%			100%		
Species	FLC	CSP	Ebselen	Ebselen	VOR	POS
C. parapsilosis[a]	0.5		0.5	4		
C. krusei[a]	16		1	4		
Paecilomyces variotii[a]			4	8		0.125
C. albicans	<0.125		2	4		
	<0.125		2	4		
	>64		2	2		
C. glabrata		1	1	4		
		0.25	0.5	1		
		0.25	1	2		
Cryptococcus neoformans	8		4	4		
	32		2	2		
	32		1	2		
Rhizopus arrhizus			8	16		0.5
			8	16		0.25
Aspergillus fumigatus			4	4	0.5	
			4	4	2	
			4	4	0.25	
Fusarium oxysporum			4	4	4	
			4	4	4	
Fusarium solani			4	8	>16	
Scedosporium apiospermum			8	8	2	
			2	8	1	
Lomentospora prolificans			4	>32	>16	
			4	>32	>16	
			2	8	>16	

[a]Quality control strains.

combinations, with the only exception being synergy in the inhibition of *C. albicans* by the combination of ebselen with fluconazole. Overall, from these experiments, we interpret the lack of antagonism as a positive finding, as this may open up new opportunities for combination treatment.

Ebselen displays a broad spectrum of antifungal actions against pathogenic yeasts and molds. We tested the activity of ebselen against a panel of medically important fungi in a preliminary assessment of the antifungal actions of this repositionable candidate. These assays were performed by the Fungus Testing Laboratory according to CLSI methodology. Regarding yeasts, the results confirmed its activity against *Candida* spp. and also demonstrated similar levels of activity against *Cryptococcus neoformans* (Table 3). One of the most pressing needs in the field of medical mycology is to develop agents with activity against difficult-to-treat mold infections. As also seen in Table 3, ebselen also demonstrated *in vitro* activity against *Aspergillus fumigatus*, *Fusarium* spp., *Scedosporium apiospermum*, *Lomentospora prolificans*, and *Rhizopus oryzae*, with MIC values generally comparable to or even lower than those observed for the current antifungal agents used in these assays for comparison purposes.

In summary, the emergence and rapid spread of *C. auris* as a nosocomial pathogen, frequently associated with multidrug resistance to different classes of antifungals, demands the rapid deployment of novel effective antifungal drugs against this formidable pathogen of increasing concern. Unlike the tortuous path of "*de novo*" drug discovery, estimated to take more than 15 years and cost more than $2 billion with high attrition rates, drug repurposing, or finding new uses for existing drugs, significantly reduces the time and cost associated with the development of novel drugs and can rapidly bring benefits to patients (26). Here, by screening a repurposing library, we

identified the potent activity of ebselen against *C. auris*. Besides this emergent pathogen, ebselen also displayed antifungal activity against all other *Candida* spp. tested, expanding on its antifungal properties from previous reports (41, 53). Furthermore, ebselen displayed a broad spectrum of antifungal actions, including difficult-to-treat molds. Although further investigations are required, altogether, these data point to the promise of ebselen as a repositionable clinical agent for the treatment of *C. auris* infections as well as for the treatment of candidiasis and possibly other fungal infections in patients refractory to therapy with conventional antifungal agents. As with any repositionable candidate, a caveat is that nonantifungal off-target effects may result in a lack of specificity and lead to adverse effects that may limit the clinical utility of ebselen for the treatment of fungal infections.

MATERIALS AND METHODS

Chemical library. The Prestwick Chemical library (Prestwick Chemical, France) contains approximately 1,280 chemically and pharmacologically diverse compounds, mostly off-patent approved drugs used for the treatment of a variety of diseases and approximately 5% of other drugs at different stages of development, all with known bioavailability and safety in humans. The compounds in the library are provided in 96-well microtiter plates as 10 mM solutions in dimethyl sulfoxide (DMSO). An initial 1:100 dilution for each compound was prepared by pipetting 2 μl of this concentrated solution into 198 μl of phosphate-buffered saline (PBS) using the wells of presterilized, polystyrene, round-bottomed 96-well microtiter plates (Corning Incorporated, Corning, NY) and was stored as working stock solutions at −20°C. For follow-up experiments, pharmaceutical grade ebselen was commercially purchased from AdipoGen (San Diego, CA, USA).

Strains and culture conditions. The *C. auris* panel was obtained from the U.S. Centers for Disease Control and Prevention (30). From this panel, the multidrug-resistant *C. auris* 0390 clinical isolate was used for initial experiments, including primary screening. This isolate is resistant to azoles and amphotericin B and shows decreased susceptibility against echinocandins. Nine other *C. auris* isolates, one *C. lusitaniae*, and one *C. krusei* isolate from the CDC panel were used in follow-up experiments, as well as the *C. albicans* SC5314 type strain and clinical isolates representative of different *Candida* species, including *C. dubliniensis*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*, which were obtained from the Fungus Testing Laboratory at the University of Texas Health Sciences Center at San Antonio.

Overnight cultures of the strains were grown by inoculating cells in 20 ml of yeast extract-peptone-dextrose (YPD) (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 2% [wt/vol] dextrose) liquid medium in 150-ml flasks and incubating in an orbital shaker (150 to 180 rpm) at 30°C. After 18 h, the cells were washed with PBS and counted with a hemocytometer. The cells were then adjusted to the desired final density (typically 0.5×10^3 cells/ml for planktonic testing and 1×10^6 for biofilm testing) in RPMI medium supplemented with L-glutamine (Cellgro, Manassas, VA) and buffered with 165 mM morpholinepropanesulfonic acid ([MOPS] Thermo-Fisher Scientific, Waltham, MA) at pH 6.9.

Primary screening for inhibitors of *C. auris*. The initial screening for compounds with antifungal activity against *C. auris* was performed according to the CLSI document M27-A3 for antifungal susceptibility testing of yeasts with minor modifications (54). Briefly, the inoculum of *C. auris* strain 0390 was prepared and added to wells in the 96-well microtiter plates, each containing an individual compound from the Prestwick library at a final concentration of 20 μM. The plates were then incubated at 35°C and the MICs were read visually (for >50% inhibition) at 24 and 48 h. At the end of the 48-h incubation period, the cells in the wells were homogenized and the absorbance determined spectrophotometrically with a microtiter plate reader to provide a more quantitative measure of inhibition. The first column of the plate contained only RPMI medium without drugs and the last column contained no cells, which served as negative and background controls, respectively. The screening was performed in duplicates. Compounds found to inhibit greater than 70% of planktonic growth (based on absorbance readings at 48 h) were initially selected as "hits."

Dose-response assays for confirmation of initial hits. The confirmation of the antifungal activity of drugs that were identified as hits from the initial (i.e., primary) screening was performed by dose-response assays using broth microdilution techniques according to CLSI methodology (54). The starting concentration of the hits was 40 μM, and serial 2-fold dilutions were performed across the rows of the 96-well microtiter plates to 0.078 μM. Positive and negative controls were also included, and experiments were performed in duplicates at each dose in two different plates. The plates were incubated for 48 h, after which the cells in the wells were homogenized and the absorbance determined with a microtiter plate reader. To prepare the dose-response curve, the spectrophotometric readings were converted into normalized responses, for which values from positive control (untreated) wells and negative control (uninoculated) wells were arbitrarily set as 100% and 0% growth, respectively. After this, the IC_{50}s, defined as the concentration of drug required to reduce planktonic growth by half, were determined by fitting the normalized values to the variable slope Hill equation (an equation determining the nonlinear drug dose-response relationship) using Prism (GraphPad Software Inc., San Diego, CA).

Determination of the antifungal activity of ebselen against different *Candida* spp. The *in vitro* antifungal activity of ebselen against *C. auris* strains in the CDC panel, *C. albicans* SC5314 type strain, and different clinical isolates representing different *Candida* spp. was determined by using the same assay, based on CLSI document M27-A3 for yeast susceptibility testing, described above for dose-response

experiments, and using spectrophotometric readings. For these experiments, the range of ebselen concentrations tested was from 32 to 0.0625 μg/ml. IC_{50}s were determined as described above.

Activity of ebselen against biofilms of *C. auris* isolates and other *Candida* spp. We used the 96-well microtiter plate model of *Candida* biofilm formation previously developed by our group to test the activity of ebselen for the prevention of biofilm formation of *C. auris* and other species (50, 55). Briefly, to evaluate the effect of the drugs in preventing biofilm formation, 50 μl of ebselen diluted in RPMI medium to appropriate concentrations (ranging from 32 μg/ml to 0.0625 μg/ml) was added to 96-well plates containing 50 μl of 2×10^6/ml of yeast cells, and the plates were incubated at 37°C for an additional 24 h. The plates were then washed once with PBS to remove nonadherent cells. The extent of biofilm inhibition was estimated with a colorimetric assay on the basis of the reduction of XTT (Sigma, St. Louis, MO) by metabolically active cells as previously described by us. From these, the IC_{50}s were determined as described above, but this time using the XTT colorimetric readings. For comparison purposes, the same assays were used in parallel to determine the activity of current antifungals (fluconazole, amphotericin B, and caspofungin) against *C. auris* biofilms. For these studies, a stock solution of fluconazole (Hospira, Lake Forest, IL) was prepared in sodium chloride for injection at 2 mg/ml and stored at 4°C. Amphotericin B was obtained in solution at 250 μg/ml (Gibco Life Technologies, Grand Island, NY) and stored at −20°C. Caspofungin (Merck & Co., Inc., Whitehouse Station, NJ) was obtained as a powder and stored at 4°C; a stock solution was prepared in PBS at 2 mg/ml the day it was added to the plates.

An alternative method was used to assess the biofilm biomass of the 10 *C. auris* isolates, in which the biofilms were stained with crystal violet as previously described (56). Briefly, after biofilm formation, the plates were washed once with PBS, and each well was treated with 100 μl of methanol for 20 min for fixation. The methanol was removed, and the plates were allowed to dry. Adherent biofilms were stained for 10 min with 150 μl of 3% (wt/vol) crystal violet. After crystal violet was removed and the plates allowed to dry, they were washed 3 times with 200 μl of distilled water. For microscopy, the stained samples were directly observed on the 96-well plate by using a 40× objective on an inverted microscope system (Westover Scientific, Mill Creek, WA) equipped for photography. The images were processed for display using Micron software (Westover Scientific). To measure biomass, 100 μl of 33% glacial acetic acid was added to each well to dissolve the dye after microscopy. Glacial acetic acid was left in the wells for 5 min while the plates were slowly shaken. The solution was then transferred to a new microtiter plate for optical density at 550 nm (OD_{550}) measurements to calculate the extent of biofilm inhibition relative to untreated controls.

Drug combination studies with ebselen and clinically used antifungals. We assessed the efficacy of combinations of ebselen together with fluconazole, caspofungin, and amphotericin B by checkerboard assays. Briefly, 2-fold serial dilutions of the clinically used antifungal were placed from rows A to G of a 96-well microtiter plate, whereas 2-fold serial dilutions of ebselen were placed from columns 1 to 9 of the same plate. In this scheme, column 10 contains the antifungal by itself, while row H contains ebselen alone. Appropriate positive (no drug) and negative (no organism) controls were in columns 11 and 12. Combination studies were performed for both *C. auris* and *C. albicans* under planktonic conditions and for the inhibition of biofilm formation (using the methods described above). To assess whether each combination of drugs resulted in synergistic, indifferent, or antagonistic effects, the fractional inhibitory concentration index (FICI) was used, defined as $MIC_{AB}/MIC_A + MIC_{BA}/MIC_B$. This calculation takes the MIC of each drug when mixed with the other and divides it by the MIC of the drug by itself. FICI values of $\leq$0.5 indicate synergy, indifference is defined as $>$0.5 to $\leq$4.0, and antagonism is defined as $>$4.0 (57).

Determination of the spectrum of antifungal action of ebselen. Antifungal susceptibility testing against a variety of medically important fungi was performed by the Fungus Testing Laboratory in accordance with the CLSI M27-A3 and M38-A2 reference standards for antifungal susceptibility testing for yeast and molds, respectively (54, 58). All clinical fungal isolates tested form part of the collection available in the Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01084-18.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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