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Original Article

Identification of disulfiram as a potential antifungal drug by screening small molecular libraries

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ABSTRACT

Objectives: *Candida albicans* and *Candida auris* strains are common causative species of Candidiasis. The limited number of antifungal drugs and the current situation of resistance to existing antifungals force us to search for new antifungal alternatives.

Methods: In this work, primary screening of small molecule libraries (Metabolism Compound Library and Epigenetics Compound Library) consisting of 584 compounds against *Candida albicans* SC5314 was performed. The dose-response assays, XTT assays, scanning electron microscopy and confocal laser scanning microscopy were used to confirm the antifungal activities of the selected compounds against *Candida* strains.

Results: Through the primary screening, we identified five compounds (U73122, disulfiram, BSK805, BIX01294, and GSKJ4) that inhibited strains growth $\geq$ 80% for dose-response assays. Disulfiram was identified as the most potent repositionable antifungal drug with 50% growth inhibition detected at a concentration as low as 1 mg/L. The further results showed the antifungal activity of disulfiram against biofilm formation of *Candida* strains with a 50% minimum inhibitory concentration ranging from 32 to 128 mg/L. Further observations by scanning electron microscopy and confocal laser scanning microscopy confirmed the destruction of biofilm architecture and the change of biofilm morphology after being exposed to disulfiram.

Conclusion: The study indicated the potential clinical application of disulfiram as a promising antifungal drug against candidiasis.

All authors meet the ICMJE authorship criteria. Heyu Zhang and Xuejun Ge designed the study. Weifeng Hao, Dan Qiao and Ying Han performed the screening experiment. Weifeng Hao, Ning Du, Xuefen Li and Yufeng Fan performed functional assays. Weifeng Hao, Heyu Zhang and Xuejun Ge wrote the manuscript.

1. Introduction

Candidiasis is the most common acquired opportunistic infection, with *C. albicans* being the major cause of candidiasis accounting for more than half of all cases [1]. In recent decades, the number of infections caused by non-*albicans Candida* species has also increased, especially infections due to the emerging pathogen *C. auris* [2]. Since its first report in 2009, *C. auris* has caused serious infections globally [3,4]. *C. auris* was reported to form lesser biofilm mass and exhibit poorer adherence to catheters than *C. albicans*; however, it persists on dry and moist surfaces for at least 7 days making it easily transmissible between patients, across hospitals, and in the environment [4–6]. Previous studies demonstrated that clinical isolates of *C. auris* had a high tolerance to major classes of clinically used antifungal drugs. For example, 90% of *C. auris* strains were found to be resistant to fluconazole, 30% to amphotericin B,

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and more than 5% to echinocandins [4]. In several cases, some *C. auris* strains were resistant either to fluconazole and amphotericin B or to all three antifungal drugs [4,7].

It is recommended that patients infected or colonized with *C. auris* be first treated with echinocandins due to the relatively low resistance [8]. However, recent studies have demonstrated that the utility of echinocandins is limited because of drug-drug interactions and a high level of liver toxicity [9]. There is an urgent need for newer antifungal agents active against *C. auris*. However, the development of entirely new pharmaceutical agents is extremely expensive and time-consuming due to the arduous approval processes [10]. Thus, we used here a more efficient alternative to the conventional bench-to-bedside drug development process — repurposing existing small compounds for antifungal activity. We screened the Metabolism Compound Library (MCL) and Epigenetics Compound Library (ECL) and found that several of them (especially disulfiram) had well-characterized pharmacological properties for development into possible antifungal agents.

2. Materials and methods

2.1. Drug libraries

The MCL and ECL were purchased from Selleck (Houston, Texas, USA). The MCL contains 403 small molecules possessing numerous biological activities via various metabolic pathways. The ECL contains 181 compounds with biological activity relevant to epigenetic research. More than half of these compounds have been approved by the Food and Drug Administration (FDA). The compounds were provided in 96-well plates as 10 mM solutions in dimethyl sulfoxide (DMSO) or water and stored at −20 °C. For follow-up experiments, 100 mM disulfiram was purchased from the same company (Selleck).

2.2. Strains and culture conditions

Strains of *C. albicans* SC5314, *C. albicans* ATCC90028, *C. auris* CBS10913, and *C. auris* CBS12373 were used in this study. *C. albicans* SC5314 and *C. albicans* ATCC90028 were obtained from the American Type Culture Collection (ATCC). *C. auris* CBS12373 and *C. auris* CBS10913 were from Jianbin Wang Laboratory in Tsinghua University and Singapore A*-STAR IMCB Yue Wang laboratory. Isolates were maintained on yeast extract-peptone-dextrose (YPD) agar medium (Hopebiol, Qingdao, China) before propagation in YPD medium for 16–20 h at 30 °C with agitation (200 rpm). Stock solution (50 mg/mL) of fluconazole (FLC, YuanYe, Shanghai, China) in DMSO was stored at −20 °C.

2.3. Primary screening of compounds against C. albicans

C. albicans SC5314 was used for primary screening of compounds. The experiment followed the EUCAST methodology with minor modifications [11]. The inoculum of *C. albicans* SC5314 was added to 96-well microtiter plates (Corning Incorporated, Corning, NY), and the cell density was adjusted to 1×10^5 cells/mL using RPMI 1640 medium supplemented with L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), and buffered with 165 mM morpholinepropanesulfonic acid (Sigma-Aldrich, St. Louis, MO, USA) at pH 7.0. All compounds were tested at a fixed concentration of 133 μM. After the plates were incubated for 48 h at 37 °C, growth inhibition was evaluated by spectrophotometrical readings (BIO TEK, Vermont, USA) at 630 nm. Compounds that inhibited the growth of planktonic by at least 80% were identified as “hits”.

2.4. Dose-response assays

The antifungal activity of the compounds identified as hits in the primary screening was confirmed by dose-response assays [11]. Briefly, drug solutions at concentrations ranging from 0.125 mg/L to 32 mg/L were prepared in RPMI 1640 medium using the serial 2-fold dilutions procedure; 50 μL of each solution was added to plates containing 50 μL of 2×10^5 cells/mL in each well. After the plates were incubated for 48 h at 37 °C, growth inhibition was evaluated by spectrophotometrical readings at 630 nm. DMSO at 1% (v/v) final concentration was added as negative control to each assay plate. The MIC_{50} and MIC_{80}, defined as the lowest concentration of drug that inhibited at least 50% and 80% of cell growth, respectively, were determined. To compare drug activity, FLC was used as a positive control for the assay.

2.5. Effect of disulfiramon Candida biofilm formation

Previously described biofilm formation assay was performed using 96-well microtiter plates [12]. Disulfiram was prepared at concentrations from 8 mg/L to 512 mg/L in RPMI 1640 medium; 50 μL of each drug solution was added to a 96-well plate containing 50 μL of 2×10^7 cells/mL per well. After the plates were incubated for 48 h at 37 °C, the effect of disulfiram on biofilm formation was evaluated using the 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2Htetrazolium-5-carboxanilide (XTT, Macklin, Shanghai, China) assay, and the plate was read spectrophotometrically at 490 nm [12]. The $BMIC_{50}$ and $BMIC_{80}$, defined as the lowest concentration of drug that inhibited at least 50% and 80% biofilm formation, respectively, were determined.

2.6. Scanning electronic microscopy (SEM)

The effect of disulfiram on *Candida* biofilm formation was examined by treating *C. auris* and *C. albicans* (1×10^7 cells/mL) with 64 mg/L and 128 mg/L disulfiram, respectively. After incubation for 48 h at 37 °C, the biofilm was washed twice with PBS, fixed with 2.5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 4 °C, and dehydrated successively with gradient alcohol (50%, 70%, 80%, 90%, 95%, and 100%) for 10 min. Finally, the samples were dried for 30 min and visualized under a SU8000 scanning electron microscope (Hitachi, Tokyo, Japan) at 5 kV [13].

2.7. Confocal laser scanning microscopy (CLSM)

Biofilm formations by *Candida* on microscope cover glass (Nest, Wuxi, China) placed in a 24-well microtiter (Corning Incorporated, Corning, NY) were prepared by incubating for 48 h at 37 °C, washing twice with PBS, and staining with carboxyfluorescein succinimidyl ester (CFSE, Selleck) at 2.8 mg/L for 20 min covered with tinfoil to keep it in dark [14]. The samples were rewashed twice with PBS and visualized under a confocal laser scanning microscope (Carl Zeiss, Jena, Germany) using an Argon laser (excitation 488 nm; emission 500–550 nm) [15]. At least four random positions were measured for each sample.

2.8. Statistical analysis

The statistical analysis of all data was performed using SPSS 23.0 software (Chicago, USA) to determine significant differences between treated and control groups by one-way analysis of variance. $P < 0.05$ was considered statistically significant. Except for the primary screening of compounds against *C. albicans*, which was performed in duplicate, other experiments were performed in triplicate.

3. Results

3.1. Screening the libraries for inhibitors of C. albicans growth

We identified 42 candidates that showed greater than 80% fungal-growth inhibition in the primary screening of the compounds listed in the MCL and ECL (Table 1). The presence of several known antifungal drugs (fluconazole, voriconazole, and ketoconazole) among the screened compounds indicates the validity and rationality of our screening technique. We generated a prominently higher hit rate than the typically reported rate of 0.1% [16], possibly because of the high drug concentration used. High drug concentrations lead to more neglected drugs being identified, many of which may be valuable.

3.2. Dose-response assays of the selected compounds

Since our main goal was to find repositionable compounds, five active compounds (U73122, disulfiram, BSK805, BIX01294, and GSKJ4) with no previous clinical indication as antifungals were selected to perform additional dose-response assays. Fig. 1 shows the dose-response effects of these five compounds. From the dose-response curves, we determined the MIC_{50} and MIC_{80} of the compounds inhibiting the growth of planktonic *Candida* strains (Table 2). It became obvious that disulfiram showed very strong activity, inhibiting at least 50% of planktonic growth, at a concentration as low as 1 mg/L. The activity of FLC against *Candida* strains was also evaluated (Table 2). *C. albicans* SC5314 and *C. albicans* ATCC90028 were sensitive to FLC. However, *C. auris* CBS10913 was moderately affected (MIC_{50} = 1 mg/L) by FLC, and *C. auris* CBS12373 exhibited high resistance to FLC (MIC_{50} = 32 mg/L). Against *C. albicans* and *C. auris* CBS10913, the remaining four compounds (MIC_{50} ranging from 4 to 16 mg/L) were not as effective as fluconazole, but their effect against *C. auris* CBS12373 (MIC_{50} ranging from 8 to 16 mg/L) was significantly more potent than FLC.

3.3. The activity of disulfiram against Candida biofilm formation

Disulfiram showed the strongest antifungal activity against all tested strains, thus, it was selected as the lead compound for follow-up screening experiments. As shown in Fig. 2, disulfiram showed dose-dependent inhibition. At a low concentration of 8 mg/L, it already showed significant antifungal activity against three biofilms of *Candida* strains ($P \leq 0.05$). The $BMIC_{50}$ and $BMIC_{80}$ results (mg/L) for disulfiram, respectively, were as follows by species: *C. auris* CBS10913 (32, 128), *C. auris* CBS12373 (32, 64), and *C. albican* SC5314 (128, >256). Our data also showed that the biofilms were highly resistant to FLC, and the $BMIC_{50}$ against *C. albicans* SC5314, *C. auris* CBS12373, and *C. auris* CBS10913 was ≥512 mg/L.

Biofilm formation was further monitored by SEM. Fig. 3A shows the SEM ultrastructure of *Candida* strains in the presence or absence of disulfiram for 48 h. The control group of *C. albicans* SC5314 showed a mature biofilm structure composed of yeast, pseudohyphae, and hyphae, consistent with other reports [17]. *C. auris* CBS12373 and *C. auris* CBS10913 did not show multiple morphologies, growing only in elliptical, smooth, and regular yeast forms, as previously described [18,19]. After treatment with disulfiram, wrinkled and aggregated yeasts were seen in *C. albicans* SC5314. Similar morphological changes in *C. auris* CBS12373 and *C. auris* CBS10913 were also observed with a poor biofilm structure.

The ultrastructural changes of *Candida* biofilms visualized by SEM were further confirmed using CLSM. Fig. 3B shows a compact three-dimensional structure in the control groups. The images presented lower biofilm biomass density in the cells treated with disulfiram compared with that in the control groups of cells. Disulfiram reduced the mean thickness of the biofilms of the three *Candida* strains.

Table 1
Hit compounds at primary screening against *C. albicans* SC5314.

Hit Compounds	% Inhibition	Hit Compounds	% Inhibition
Acarbose	>95	Triclosan	89
Beta-Lapachone	>95	Roxadustat	88
BIX01294	>95	AZ6102	87
BSK805	>95	MG149	87
Clarithromycin	>95	Quisinostat	87
Disulfiram	>95	UNC669	87
Ganetespib	>95	CHIR-99021	86
GSKJ4	>95	Mesalamine	86
Ketoconazole	>95	ENMD-2076	85
Luminespib	>95	ML324	85
U73122	>95	Quinacrine 2HCl	85
Voriconazole	92	Sulfameter	85
Fluconazole	90	KW-2449	84
HSP990	90	MK-8245	84
MM-102	90	ORY-1001	84
Paeonol	90	ZM 39923 HCl	84
PX-12	90	Alvespimycin	83
UNC0379	90	Carbidopa	83
WP1066	90	gossypol-Acetic acid	81
Celastrol	89	PU-H71	81
Cladribine	89	Methyldopa	80

4. Discussion

Candidiasis is one of the most common fungal infections that is often caused by *C. albicans*. More recently described *C. auris* pathogen has exacerbated the challenge of treating candidiasis due to multidrug antifungal resistance worldwide [2]. Classic antifungal agents have various drawbacks including host liver toxicity and intrinsic resistance. Repurposing already known compounds as new antifungal agents provides an efficient and economical alternative, as reported previously by many groups [20,21]. Such methods would likely be beneficial to accelerate the drug discovery because their structure, chemical properties, and biological functions are already described in the above libraries [22].

After the initial screening, we focused on five compounds with different mechanisms of action that have previously not been referred to as antifungals in clinical use. Some studies have demonstrated that U73122 increased the activity of Phospholipase C (PLC) in a concentration-dependent manner, and PLC plays a critical role in the antifungal innate immune response [23]. Our study suggests that U73122 may be a novel compound for the development of antifungal drugs. BSK805 which acts as a selective ATP-competitive JAK2 inhibitor showed good oral bioavailability, and has been used as a representative compound in the treatment of chronic myeloproliferative neoplasms and hematologic malignancies in mouse and rat models [24]. GSKJ4 is an ethyl ester derivative of GSK-J1, the Jumonji domain-containing protein 3 selective histone demethylase inhibitor that inhibits tumor progression and embryonic stem cell differentiation [25]. BIX01294 is an inhibitor of G9a histone methyltransferase and is a potential therapeutic agent in the treatment of multistage antimalarial and myeloid leukemia [26,27]. To the best of our knowledge, the antifungal activity of these three compounds (BSK805, GSKJ4, and BIX01294) was first pointed out in our study; however, their mechanisms of action need to be researched.

As a FDA approved, long-standing drug, disulfiram has been tested in multiple clinical trials, and has played an exceptional role in the treatment of alcohol dependence for over six decades [28,29]. The main pharmacological action of disulfiram is to inhibit the activity of aldehyde dehydrogenase (ALDH) enzyme that leads

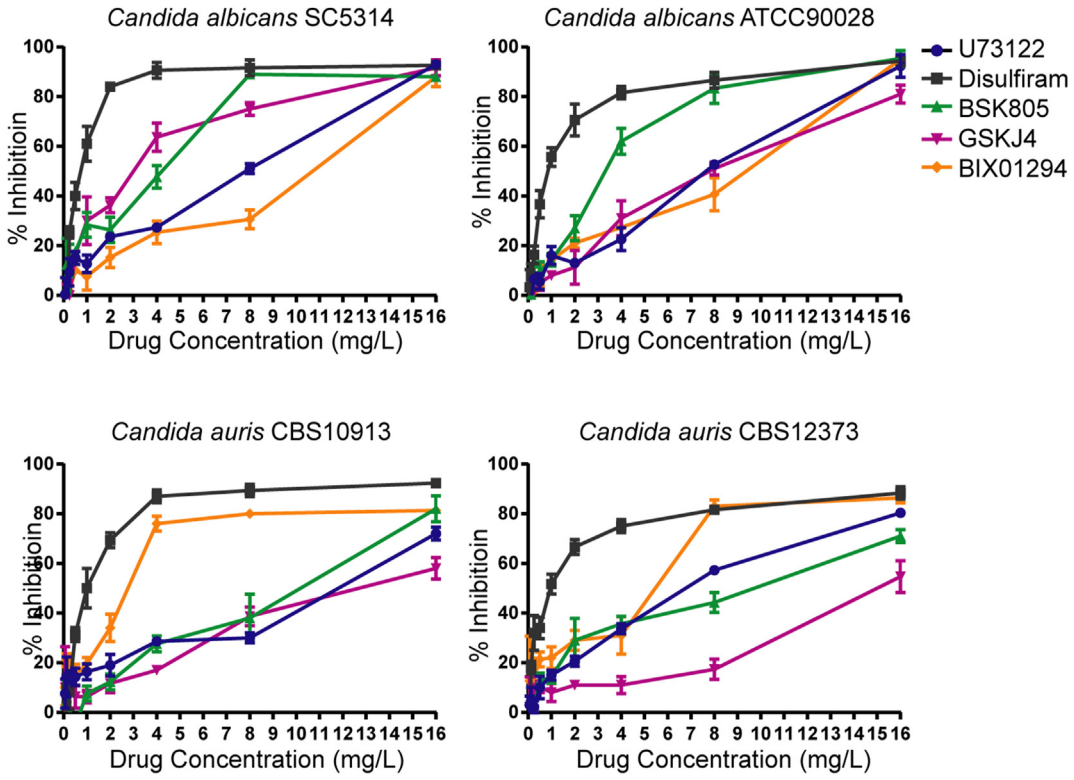

Fig. 1. Dose-response assays to confirm the inhibitory activity of five compounds. Bars indicate standard errors.

Table 2
Antifungal activity of the compounds and FLC against *Candida* strains.

Compounds (mg/L)	MIC values							
	C. albicans SC5314		*C. albicans* ATCC90028		*C. auris* CBS12373		*C. auris* CBS10913	
	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}
U73122	8	16	8	16	8	16	16	>16
Disulfiram	1	2	1	4	1	8	1	4
BSK805	8	8	4	8	16	>16	16	16
GSKJ4	4	16	8	16	16	>16	16	>16
BIX01294	16	16	16	16	8	8	4	8
FLC	0.125	0.125	0.125	0.125	32	32	1	2

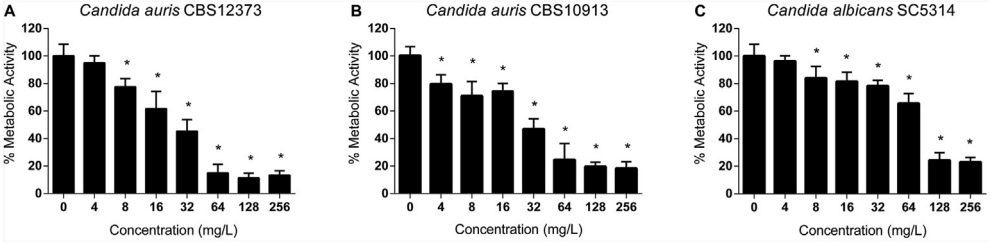

Fig. 2. Inhibitory effects of disulfiram against biofilm formation. (A) *C. auris* CBS12373; (B) *C. auris* CBS10913; (C) *C. albicans* SC5314. The metabolic activity of biofilm cells was evaluated using the XTT reduction assay. Bars indicate standard errors. *$p < 0.05$.

to negative disulfiram-ethanol reactions in patients [28,30]. Disulfiram has also been proposed as a potential anti-cancer drug, etc. [29]. The antifungal activity of disulfiram against *C. albicans* has been mentioned in a previous study and the MIC_{50} and MIC_{90} of disulfiram for the tested *C. albicans* isolates were 2 and 4 μg/ml, respectively, which were similar to our results [31]. To the best of our knowledge, our study was first to test the *anti*-biofilm activity of disulfiram against *C. albicans and* demonstrate that disulfiram exhibits *anti*-biofilm and antifungal activities against *C. auris*, particularly resistant *C. auris* CBS12373 on which disulfiram showed superior activity over FLC. In addition, *Galleria mellonella* 's study has reported that aggregating cells are less virulent and pathogenic than non-aggregating cells [19]. In our study, disulfiram increased cell aggregation and decreased biofilm formation that further supported its activity against *Candida* strains. Based on a previous report, disulfiram inhibits the activity of ATP binding

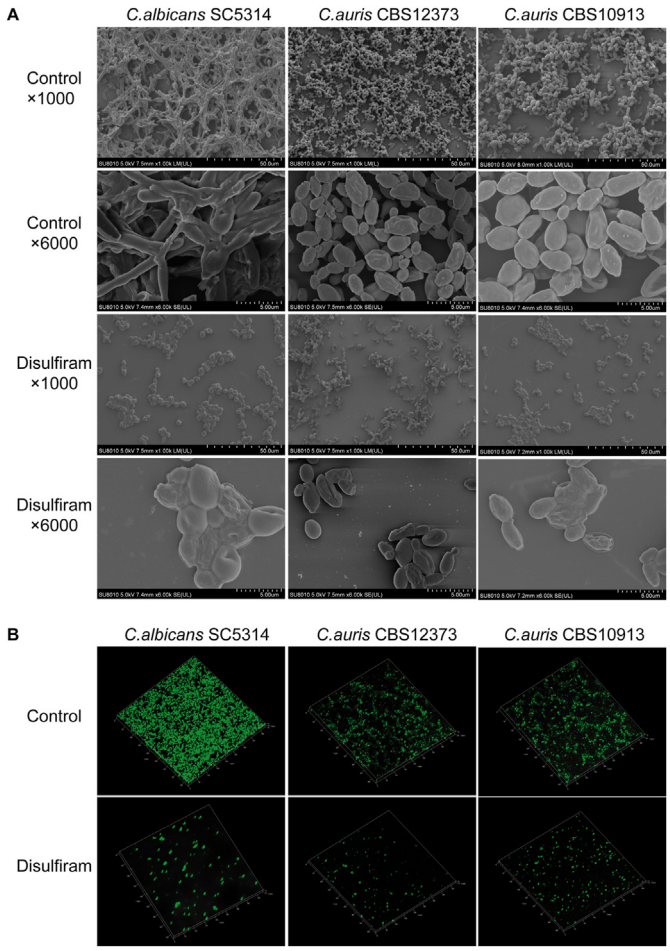

Fig. 3. The images of biofilms formed by different strains of *Candida*. Biofilms were exposed to no drug (control) or disulfiram at different concentrations: 128 mg/L (*C. albicans* SC5314), 64 mg/L (*C. auris* CBS12373 and *C. auris* CBS10913). (A) The SEM images of biofilms were showed. (B) The CLSM images of biofilms were showed. Magnification × 200.

cassette drug transport proteins that are associated with antifungal resistance [31]. Thus, disulfiram may be considered as a potential agent in the treatment of candidiasis and may play an important role in combating multiple drug resistance.

In summary, we identified five compounds that showed antifungal activity by screening compounds in the MCL and ECL. Disulfiram inhibited the growth of both planktonic and biofilm formation in the tested strains of *Candida*. These results are of great significance in finding new antifungal drugs for the treatment of *C. auris* (especially FLC resistant strains) infections. However, this study has many limitations because only a small number of *Candida* strains have been tested. Further experiments need to be performed to confirm the activity and mechanism of disulfiram alone or in combination with other antifungal drugs against candidiasis.

Declaration of competing interest

None of the authors have any Competing interest.

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