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Article

Discovery of Piperidol Derivatives for Combinational Treatment of Azole-Resistant Candidiasis

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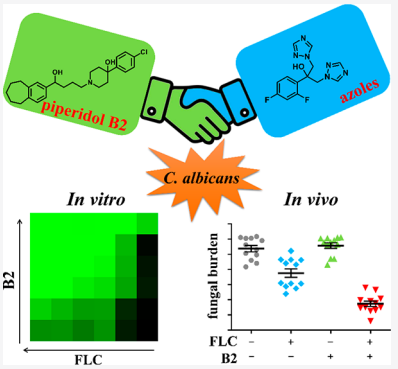
ABSTRACT: Effective strategies are needed to deal with invasive fungal infections caused by drug-resistant fungi. Previously, we designed a series of antifungal benzocyclane derivatives based on the drug repurposing of haloperidol. Herein, further structural optimization and antifungal mechanism studies were performed, leading to the discovery of new piperidol derivative **B2** with improved synergistic antifungal potency, selectivity, and water solubility. In particular, the combination of compound **B2** and fluconazole showed potent *in vitro* and *in vivo* antifungal activity against azole-resistant *Candida albicans*. Compound **B2** inhibited important virulence factors by regulating virulence-associated genes and improved the efficacy of fluconazole by down-regulating the CYP51-coding gene and efflux pump gene. Taken together, the piperidol derivative **B2** represents a promising lead compound for the combinational treatment of azole-resistant candidiasis.



KEYWORDS: *antifungal, piperidol derivatives, drug resistance, virulence factors, Candida albicans*

The morbidity and mortality of invasive fungal infections (IFIs) of at-risk patients have increased dramatically due to the limitations of current antifungal therapies.[1−3] The major pathogenic fungi of IFIs include *Cryptococcus*, *Candida*, *Aspergillus*, and *Pneumocystis*, among which *Candida albicans* (*C. albicans*) is the most frequent cause of IFIs with mortality rates of 46%−75%.[4−6] Currently, antifungal drugs used to treat IFIs are mainly divided into four classes: polyenes, azoles, echinocandins, and flucytosine, whose clinical applications have been hampered by limited antifungal potency and high toxicity.[7,8] The long-term use of a single antifungal agent has resulted in drug resistance, and the prevalence of drug resistance makes curing IFIs generally unachievable. Therefore, the development of mechanistically distinct antifungal therapies is urgently needed.

The search for synergistic enhancers of existing antifungal drugs is an effective strategy to overcome the drug resistance of *C. albicans*.[9] Azoles have been used widely to treat candidiasis since 1980s with species-selectivity toward the fungal lanosterol 14α-demethylase (CYP51). However, resistance to azoles has emerged as a serious threat to patients in the clinic due to their fungistatic feature, which involves the upregulation of CYP51-coding and efflux pump genes.[10] Recent efforts have revealed that combinational treatments were able to enhance the antifungal efficacy and specificity and block the evolution of drug resistance. For example, calcineurin inhibitors and flucytosine have been proven to synergize with fluconazole (FLC) against azole-resistant *C. albicans*.[11] However, effective enhancers of azole antifungal agents with *in vivo* synergistic potency are still rare.

Previously, our group designed a series of benzocyclane derivatives based on the drug repurposing of haloperidol (HAL) for the treatment of cryptococcosis and candidiasis (Figure 1).[12] Benzocyclane **1** showed good synergistic effects with FLC against azole-resistant *C. albicans* with a fractional inhibitory concentration index (FICI) of 0.375, which offered a promising lead compound for the development of novel synergistic enhancers. However, the structure−activity relationships (SARs) of the benzocyclane lead compound are rather limited and the underlying synergistic mechanism is still unclear. Importantly, the synergistic antifungal activity as well as solubility remains to be further improved.

Herein a series of analogues was designed by scaffold hopping of the benzocyclane skeleton and the incorporation of a hydroxyl group (or ketone) on the side chain (Figure 1). After biological evaluations, new benzocyclane derivative **B2** showed improved th esynergistic antifungal potency, selectivity, and water solubility. Moreover, synergistic mechanism investigation revealed that compound **B2** could inhibit important virulence factors by regulating virulence-associated genes and improve the efficacy of FLC by down-regulating the

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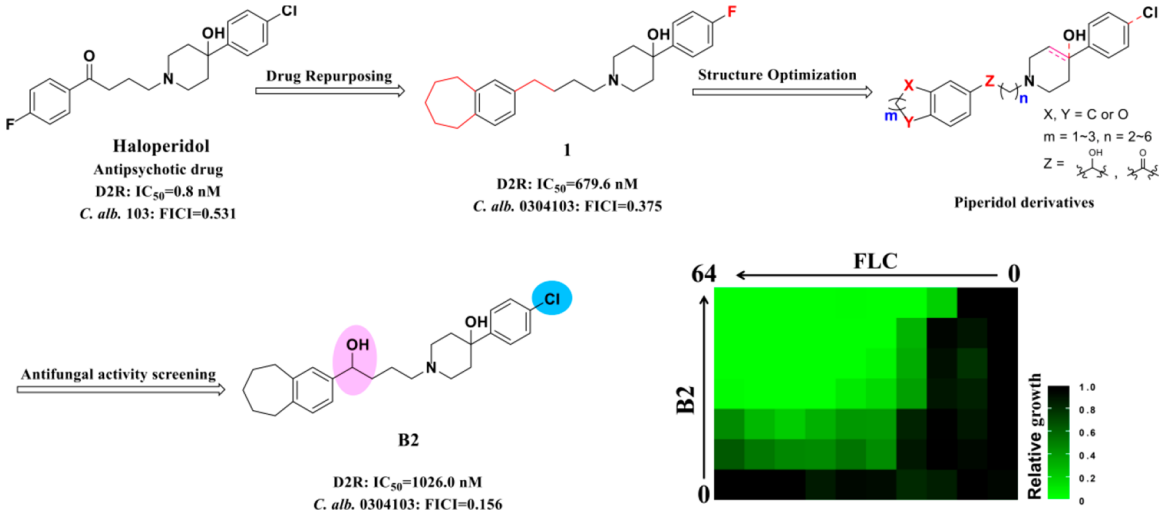
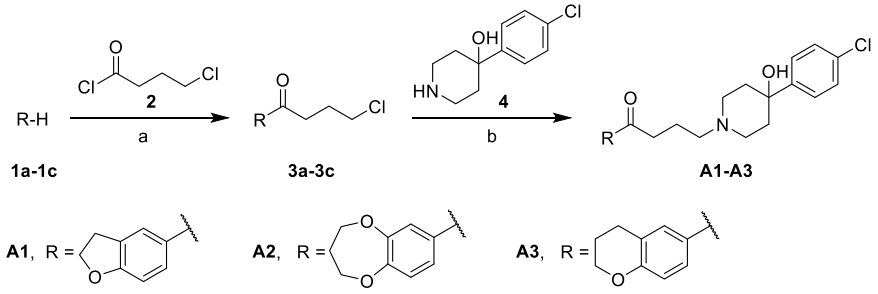


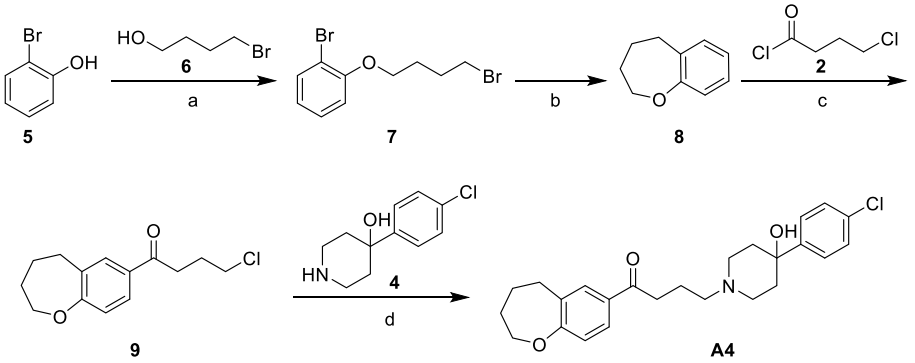
Figure 1. Design of synergistic antifungal compound **B2**. As shown in the heat map, the *in vitro* synergistic antifungal activity of compound **B2** with FLC was measured using a checkerboard microdilution method. The color changes from green to black were used to express the relative growth of the fungi (0−1.0); if the color was greener, the inhibition was stronger (FICI < 0.5, synergism; FICI > 4, antagonism; 0.5 ≤ FICI ≤ 4, indifferent).

Scheme 1. Synthesis of Compounds A1−A3



[a]Reagents and conditions: (a) $AlCl_3$, DCM, 0 °C, 1.5 h, yield 65.9%−94.1%; (b) DIPEA, DMF, 35 °C, 16 h, yield 39.4%−47.7%.

Scheme 2. Synthesis of Compound A4[a]



[a]Reagents and conditions: (a) DEAD, Ph_3P, THF, 0 °C-rt, overnight, yield 51.9%; (b) *n*-BuLi, THF, −78 °C, 1.5 h, yield 60.4%; (c) $AlCl_3$, DCM, 0 °C, 1.5 h, yield 88.1%; (d) DIPEA, DMF, 35 °C, 16 h, yield 44.2%.

expression of the CYP51-coding gene and efflux pump gene of resistant *C. albicans*.

RESULTS AND DISCUSSION

Chemistry. Two series of new piperidol derivatives (class **A** and class **B**) were designed and synthesized on the basis of the molecular skeleton of benzocyclane **1**, and the chemical synthesis is depicted in Schemes 1−4. The starting materials **1a−1c**, **2**, **4−6**, **11**, **2a−2e**, and **13a−13c** were commercially available. The intermediates **3a−3c**, **7−9**, **12a−12e**, and **14a−14d** (Schemes S1−S4 in the Supporting Information) and the target compounds **A1−A3** (Scheme 1) were prepared according to our previously reported methods.[12] The synthetic route of target compound **A4** is shown in Scheme 2. Intermediate **7** was obtained by the Mitsunobu reaction between 2-bromophenol and 4-bromobutan-1-ol in tetrahydrofuran (THF). Intermediate **7** was cyclized in the presence of *n*-butyllithium at −78 °C to afford intermediate **8**, which further reacted with 4-chlorobutanoyl chloride to form intermediate **9** (Scheme S2). Then, intermediate **9** was

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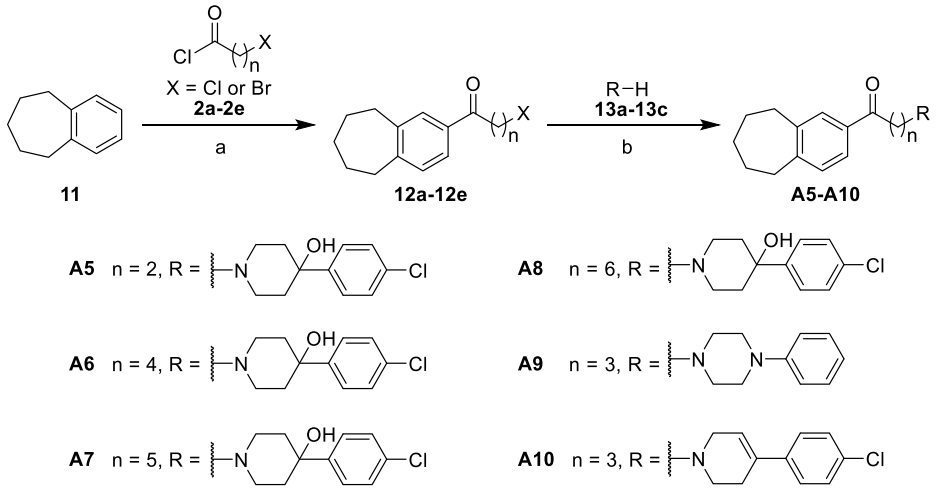
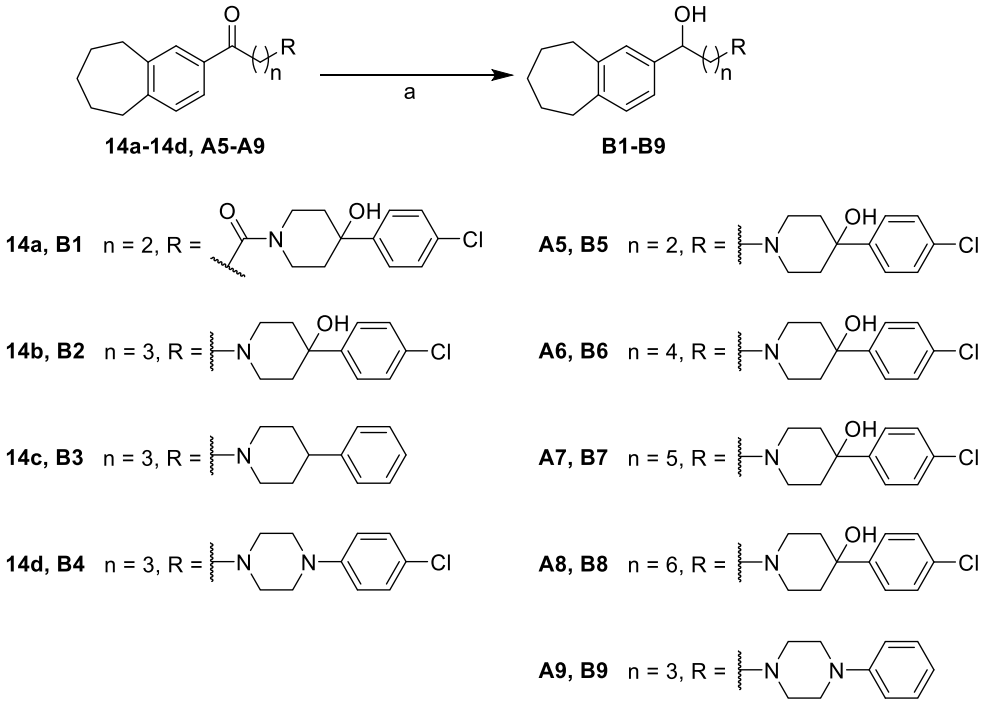
Scheme 3. Synthesis of Compounds A5−A10[a]



[a]Reagents and conditions: (a) $AlCl_3$, DCM, 0 °C, 1.5 h, yield 42.6%−80.9%; (b) DIPEA, DMF, 35 °C, 16 h, yield 34.0%−46.7%.

Scheme 4. Synthesis of Compounds B1−B9[a]



[a]Reagents and conditions: (a) $NaBH_4$, MeOH, rt, 1.5 h, yield 61.4%−81.7%.

substituted by piperidine derivative **4** to obtain compound **A4** (Scheme 2). Catalyzed by $AlCl_3$, intermediate **11** reacted with acyl chloride in dichloromethane (DCM) by Friedel−Crafts acylation to obtain intermediates **12a−12e**, which were further substituted by different amines to obtain target compounds **A5−A10** (Scheme 3).[12] As shown in Scheme 4, the target compounds **A5−A10** were reduced by using $NaBH_4$ in MeOH to obtain the target compounds **B1−B9**.

Antifungal Susceptibility Testing and SAR of Piperidol Derivatives. The antifungal susceptibility testing of piperidol derivatives **A1−A10** and **B1−B9** were measured, in which the inhibition of fungal growth was evaluated by determining the minimum inhibitory concentration with inhibition over 80% (MIC_{80}), and the synergistic activity was expressed by FICI. FLC and HAL were used as positive controls. As shown in Table 1, most piperidol derivatives were almost inactive against *C. albicans*, *C. glabrata*, *C. parapsilosis*, or *C. neoformans* when used alone. Among them, only compounds **B2**, **B5**, and **B6** showed weak inhibitory activity against *C. neoformans* (MIC_{80}: 16 μg/mL). Furthermore, the synergistic antifungal activity of compounds **A1−A10** and **B1−B9** against azole-resistant *C. albicans* was also evaluated (Table 2). SARs revealed that incorporating an oxygen atom or reducing the cycloalkyl ring size of benzocyclane scaffold (compounds **A1−A4**) generally led to decreased synergistic antifungal activity. After a ketone group was introduced on the side chain (compounds **A5−A10**), most compounds lost the synergistic effects (FICI > 0.5). Interestingly, the synergistic

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Table 1. *In Vitro* Antifungal Activity of Target Compounds (MIC_{80}, μg/mL)

compound	*C. alb.*[a] SC5314	*C. gla.*[a] 8535	*C. par.*[a] 20090	*C. neo.*[a] H99
A1	>128	>128	>128	>128
A2	>128	128	>128	>128
A3	>128	>128	>128	128
A4	>128	>128	>128	128
A5	>128	>128	>128	>128
A6	>128	>128	128	>128
A7	>128	>128	>128	128
A8	>128	>128	>128	>128
A9	>128	>128	>128	>128
A10	128	>128	64	>128
B1	>128	128	>128	>128
B2	128	32	64	16
B3	>128	128	>128	32
B4	>128	128	>128	>128
B5	128	64	32	16
B6	128	64	64	16
B7	128	32	64	32
B8	128	64	64	128
B9	>128	>128	>128	>128
HAL	>128	>128	>128	64
FLC	0.5	4	1	4

[a]Abbreviations: *C. alb.*, *Candida albicans*; *C. gla.*, *Candida glabrata*; *C.par.*, *Candida parapsilosis*; *C. neo.*, *Cryptococcus neoformans*.

activity was significantly improved when the ketone group was reduced to the hydroxyl group (compounds **B1**−**B9**). Several derivatives showed potent synergistic inhibitory activity (FICI < 0.3) when combined with FLC. Notably, compound **B2** exhibited the best synergistic antifungal efficacy against azole-resistant *C. albicans* isolates including *C. alb*. 0304103 (FICI = 0.156), *C. alb*. 4108 (FICI = 0.094), and *C. alb*. 7781 (FICI = 0.156) when used in combination with FLC. The synergistic antifungal activity of compound **B2** against various fungal strains when combined with other first-line azole drugs was further tested (Table 3). The results showed that compound **B2** showed good synergistic antifungal activity (FICI < 0.3) against drug-resistant *C. alb*. 0304103 and *C. alb*. 7781 when combined with voriconazole (VRC) or itraconazole (ITC). Interestingly, compound **B2** also exhibited potent synergistic antifungal efficacy against *C. auris* 0029 when used in combination with VRC (FICI = 0.313) or ITC (FICI = 0.188). Moreover, the hydroxyl derivative **B2** (S_w = 244.5 μg/mL) possessed significantly better water solubility (S_w) than that of the lead compound **1** (S_w = 84.7 μg/mL).

Time-Growth Curve Assay. In order to further evaluate the antifungal activity against azole-resistant *C. albicans*, the time-growth curve of compound **B2** was determined. The results showed that compound **B2** (64 μg/mL) or FLC (64 μg/mL) alone had no inhibitory effect on the growth of azole-resistant *C. albicans*. By contrast, compound **B2** was synergized with FLC and showed a potent inhibitory effect in a dose-dependent manner (Figure 2). In particular, the combination of compound **B2** (64 μg/mL) and FLC (16 μg/mL) completely inhibited the growth of azole-resistant *C. albicans* cells.

Compound B2 Inhibited Filamentation Process of Azole-Resistant *C. albicans*. The pathogenic process of fungi mainly includes surface adhesion, yeast-to-hyphae morphological transition, and tissue invasion. Filamentation and biofilm formation have been recognized as critical virulence factors of *C. albicans*, which could enhance the pathogenicity or drug resistance of *C. albicans*.[13−15] Therefore, targeting hyphae or biofilm could be a promising strategy for the development of anticandidiasis drugs. The effect of compound **B2** on the filamentation process of azole-resistant *C. albicans* was investigated. As shown in Figure 3A, 8 μg/mL compound **B2** showed better inhibitory effect on the

Table 2. *In Vitro* Synergistic Antifungal Activity of Target Compounds (MIC_{80}, μg/mL)

strains	*C.alb.*[a] 0304103					*C.alb.*[a] 4108					*C.alb.*[a] 7781				
	single		synergetic			single		synergetic			single		synergetic		
	FLC	compound	FLC	compound	FICI[a]	FLC	compound	FLC	compound	FICI[a]	FLC	compound	FLC	compound	FICI[a]
A1	>64	>64	2	32	0.531	>64	>64	4	32	0.563	>64	>64	4	32	0.563
A2	>64	>64	4	16	0.313	>64	>64	4	8	0.188	>64	>64	4	16	0.313
A3	>64	>64	8	32	0.625	>64	>64	4	16	0.313	>64	>64	4	16	0.313
A4	>64	>64	16	32	0.75	>64	>64	4	16	0.313	>64	>64	8	32	0.625
A5	>64	>64	>64	>64	>2	>64	>64	4	32	0.563	>64	>64	4	32	0.563
A6	>64	64	2	32	0.531	>64	32	4	32	0.563	>64	32	4	16	0.563
A7	>64	64	8	32	0.625	>64	>64	4	8	0.188	>64	>64	32	32	1
A8	>64	64	4	32	0.563	>64	>64	2	32	0.531	>64	>64	8	16	0.375
A9	>64	>64	>64	>64	>2	>64	>64	>64	>64	>2	>64	>64	>64	>64	>2
A10	>64	>64	64	64	2	>64	>64	2	32	0.531	>64	64	4	32	0.563
B1	>64	>64	>64	>64	>2	>64	>64	>64	>64	>2	>64	>64	4	32	0.563
B2	>64	>64	2	8	0.156	64	64	2	4	0.094	>64	>64	2	8	0.156
B3	>64	>64	>64	>64	>2	>64	64	2	8	0.156	>64	>64	2	8	0.156
B4	>64	>64	>64	>64	>2	>64	>64	>64	>64	>2	>64	>64	8	32	0.625
B5	>64	64	2	8	0.156	64	64	1	8	0.141	>64	64	4	8	0.188
B6	>64	64	2	8	0.156	>64	64	2	8	0.156	>64	64	2	16	0.281
B7	>64	>64	2	8	0.156	64	64	2	8	0.156	>64	>64	1	16	0.266
B8	>64	32	8	8	0.375	64	32	2	8	0.281	>64	>64	16	8	0.375
B9	>64	>64	>64	>64	>2	>64	>64	4	32	0.563	>64	>64	4	32	0.563
HAL	64	>64	2	32	0.531	>64	>64	0.5	16	0.258	>64	>64	4	32	0.563

[a]Abbreviations: *C. alb.*, *Candida albicans*. FICI < 0.5, synergism; FICI > 4, antagonism; 0.5 ≤ FICI ≤ 4, indifferent.

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Table 3. Synergistic Antifungal Activity of B2 in Combination with Different First-Line Azole Drugs against Various Fungal Strains

	VRC		ITC		FLC	
strains	MIC_{80}(μg/mL)	FICI	MIC_{80}(μg/mL)	FICI	MIC_{80}(μg/mL)	FICI
C.alb.[a] 0304103	>64	0.129	>64	0.254	>64	0.156
C.alb.[a] 7781	>64	0.129	>64	0.141	>64	0.156
C. gla.[a] 4408	0.5	0.563	8	0.375	8	0.625
***C. neo.*[a] H99**	0.0313	0.625	0.25	1	2	0.625
C. aur.[a] 0029	64	0.313	64	0.188	>64	1
C. aur.[a] 0030	2	0.625	16	0.5	>64	1
C. aur.[a] 15448	0.0313	0.625	0.001	1.063	4	0.625

[a]Abbreviations: *C. alb.*, *Candida albicans*; *C. gla.*, *Candida glabrata*; *C. neo.*, *Cryptococcus neoformans*; *C. aur.*, *Candida auris*.

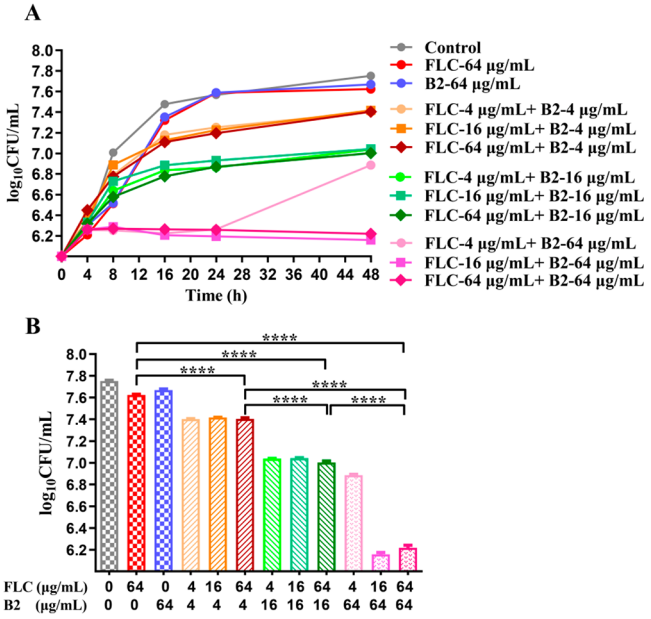

Figure 2. (A) Time-growth curve of azole-resistant *C. albicans* treated with increasing concentrations of FLC, **B2**, or their combination. (B) Statistical comparison between different treatment groups at 48 h (**** $P < 0.0001$, determined by Student's t test).

filamentation of azole-resistant *C. albicans* than 64 μg/mL FLC. Interestingly, compound **B2** (16 μg/mL) used alone could completely inhibit the filamentation as well as in combination with FLC, and the inhibitory effect was further confirmed by laser confocal microscopy (Figure 3B).

Compound B2 Inhibited the Azole-Resistant *C. albicans* Biofilms. As an important virulence factor of *C. albicans*, biofilm prevents drugs from penetrating into the cell membrane to exert antifungal activity, which results in the enhancement of drug resistance and the decrease or even loss of drug efficacy.[14,16] Therefore, the inhibitory effect of **B2** on azole-resistant *C. albicans* biofilms was further investigated. As shown in Figure 3C, the yellow of the XTT/menadione solution gradually lightened and clarified with the increase of the dose of compound **B2**. The results indicated that compound **B2** showed a well-inhibitory effect on the biofilm formation of azole-resistant *C. albicans* in a dose-dependent manner when used alone or in combination with FLC. The inhibition rate of biofilm formation was over 80% at 64 μg/mL and over 20% at 16 μg/mL when compound **B2** was used alone. In particular, the inhibitory activity of compound **B2** on biofilm formation was further improved when it was combined with FLC (16 or 32 μg/mL). The inhibition rate of biofilm formation was over 95% at 64 μg/mL and over 80% at 16 μg/mL. In addition, compound **B2** also exhibited potent synergistic inhibitory effect on biofilm formation at a lower concentration (0.25 μg/mL) when combined with FLC (16 or 32 μg/mL). In addition, the results of mature biofilm destruction assay showed that compound **B2** could destruct the preformed mature biofilm when used alone or in combination with FLC (Figure S1 in the Supporting Information).

Compound B2 Inhibited the Biofilm Formation of Azole-Resistant *C. albicans* by Regulating Biofilm Formation-Related Genes. To further explore the mechanism for the inhibition of virulence factors of *C. albicans*, real-time RT-PCR analysis was used to assess the regulation of compound **B2** on the expression levels of biofilm formation-related genes when used alone or synergistically. *BCR1*, *EAP1*, *HWP1*, *ALS3*, and *EFG1* genes encode cell wall protein adhesin and participate in biofilm formation.[17−20] *CPH1*, *ACE2*, and *TEC1* genes encode transcription factors, which are required for hyphal formation and biofilm development.[21−23] *RLM1*, *ZAP1*, *CSH1*, *ADH5*, *GCA1*, *GCA2*, and *IFD6* genes encode transcription factors, which regulate the production of extracellular matrix in biofilm.[23−26] Among them, *RLM1*, *ADH5*, *GCA1*, and *GCA2* genes are positive regulators of matrix production, while *ZAP1*, *CSH1*, and *IFD6* genes play a negative feedback regulation role.

As shown in Figure 4A, when azole-resistant *C. albicans* cells were treated with compound **B2** (16 μg/mL), the gene expressions of *ADH5* and *GCA2* were significantly down-regulated and the gene expressions of *ZAP1*, *CSH1*, and *IFD6* were significantly up-regulated as compared to the control. As compared to the FLC-treated group, the expression levels of 12 genes (i.e., *BCR1*, *EAP1*, *HWP1*, *ALS3*, *EFG1*, *CPH1*, *ACE2*, *TEC1*, *RLM1*, *ADH5*, *GCA1*, and *GCA2*) were significantly down-regulated (Figure 4B) under the synergistic action of compound **B2** (16 μg/mL) and FLC (16 μg/mL). Taken together, the results of real time RT-PCR were well-consistent with the fact that compound **B2** inhibited of azole-resistant cell wall protein adhesin biofilms when used alone or combined with FLC.

Microscopic Observation of Fungal Morphology. The morphological changes of azole-resistant *C. albicans* cells were evaluated by transmission electron microscopy (TEM) to investigate the antifungal mechanism of compound **B2**. The normal azole-resistant *C. albicans* cells have an uniform central density with clear and complete cell membranes and cell walls (Figure 5A). FLC could mildly damage the cell membranes of azole-resistant *C. albicans* cells (Figure 5C), while the cell

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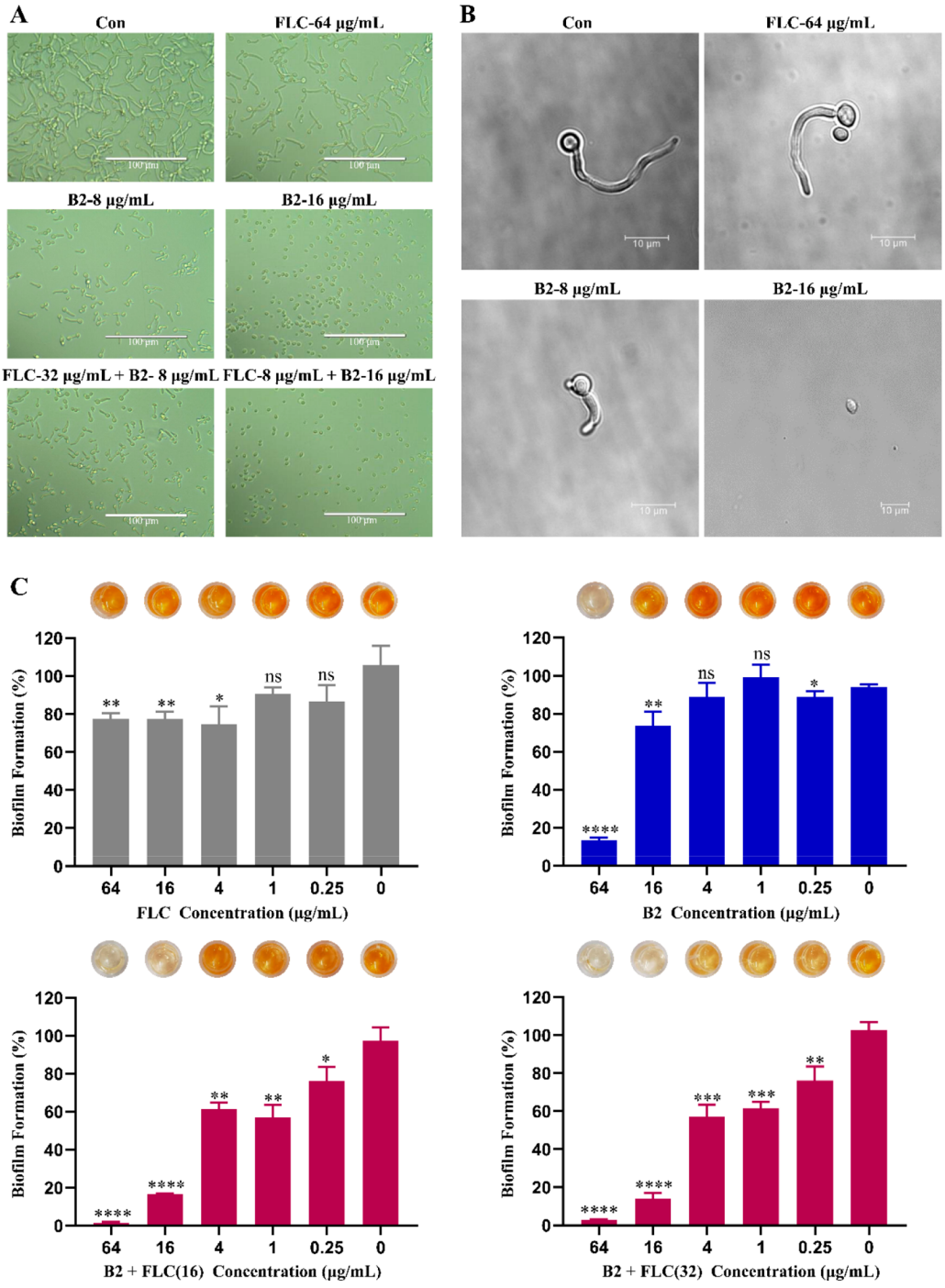

Figure 3. Treatment of FLC, compound **B2**, or both impaired the filamentation of azole-resistant *C. albicans*. The data was obtained from (A) the cell inverted imaging microscope (scale bar: 100 μm) and (B) the laser confocal microscope (scale bar: 10 μm). (C) Inhibitory effect of FLC, compound **B2**, or both on the biofilms of azole-resistant *C. albicans*. The 2,3-bis(2-hydroxyethylthio) naphthalene-1,4-dione (XTT) reduction assay was used to evaluate the inhibitory activity. The results were presented as biofilm formation percentages compared to the untreated groups and also directly expressed by the yellow degree of XTT; the lighter the yellow, the stronger the inhibition (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, determined by Student's t test).

membranes remain intact when compound **B2** was used alone (Figure 5B). In contrast, the cell membranes were obviously damaged, resulting in the release of cell contents, when treated with the combination of **B2** and FLC (Figure 5D).

Compound B2 Inhibited the Azole-Resistant *C. albicans* by Down-Regulating the Expression of *ERG11* and *CDR1*. Azole resistance has become a serious problem affecting the therapeutic effect of IFIs in clinical treatment. The high expression of *ERG11* and efflux pump genes are two important factors leading to the drug resistance of *C. albicans*.[27,28] CYP51 is the target of azole antifungal drugs, encoded by *ERG11* gene and involved in the biosynthesis of ergosterol. The mutation or high expression of *ERG11* gene in *C. albicans* would reduce the affinity of azole antifungal drugs to CYP51, thus increasing the resistance of *C. albicans*.[28,29] *CDR1* is an efflux pump gene associated with azole resistance,

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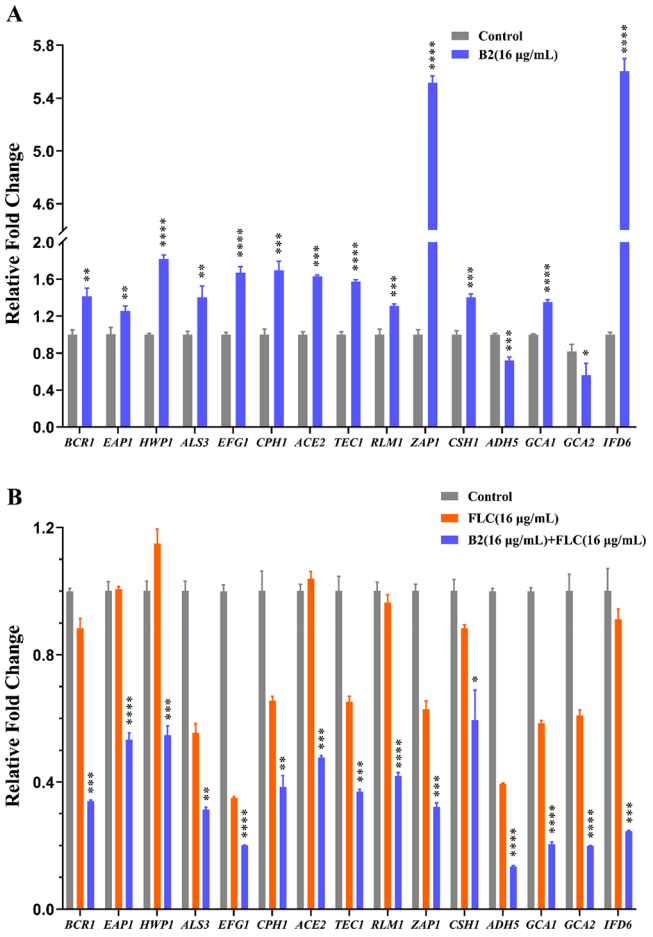

Figure 4. Regulation of (A) compound **B2** (16 μg/mL) alone or (B) combined with FLC (16 μg/mL) on the related genes of hyphae and biofilm formation (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, determined by Student's t test).

and the high expression of *CDR1* would increase the efflux of azole drugs, which reduces drug concentration in fungal cells and leads to the increased drug resistance of *C. albicans*.[30,31]

In order to further explore the antifungal mechanism of compound **B2** in inhibiting azole-resistant *C. albicans*, we investigated the regulatory effect of compound **B2** on *ERG11* and efflux-pump-related genes of azole-resistant *C. albicans* through real-time RT-PCR analysis. In comparison with the FLC-treated group, the expressions of *ERG11* ($P < 0.0001$) and *CDR1* ($P < 0.001$) genes were significantly down-regulated (Figure 6) when azole-resistant *C. albicans* was treated with compound **B2** (8 μg/mL) and FLC (8 μg/mL). The results indicated that the combination of compound **B2** and FLC could significantly inhibit the expression levels of resistant-associated genes (*ERG11* and *CDR1*), which might be the cause of the weakened resistance of *C. albicans* to FLC.

Inhibitory Activity of Compound B2 toward Dopamine D2 Receptor. HAL is widely used as an antipsychotics, which acts by targeting dopamine D2 receptor (D2R).[32,33] In order to further investigate whether compound **B2** and HAL bind to the same molecular target, the generation of cyclic adenosine monophosphate (cAMP) was detected by homogeneous time-resolved fluorescence (HTRF) assay.[34] As compared to risperidone (RIS, IC_{50} = 1.5 nM) and HAL (IC_{50} = 0.8 nM), the effect of compound **B2** (IC_{50} = 1026.0 nM) to inhibit D2R was dramatically reduced by more than 680 times

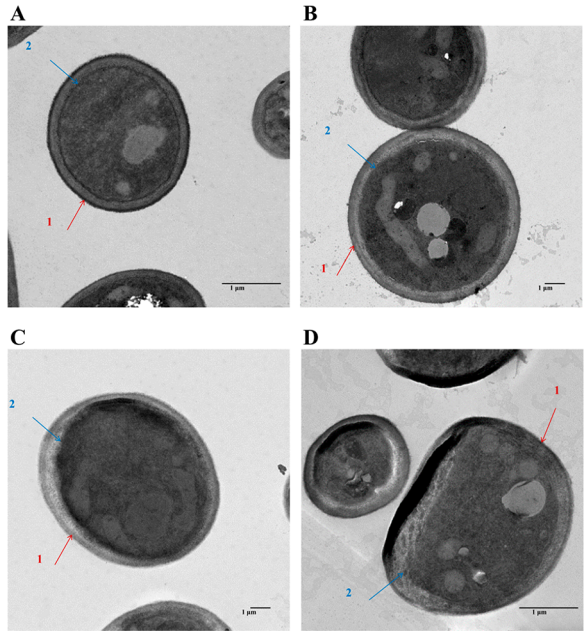

Figure 5. Morphological changes of azole-resistant *C. albicans* treated with (A) DMSO, (B) **B2** (4 μg/mL), (C) FLC (16 μg/mL), or (D) **B2** (4 μg/mL) + FLC (16 μg/mL), which were observed by transmission electron microscopy. The arrows indicate structures as follows: (1) cell wall and (2) cell membrane.

and 1200 times, respectively. The results suggested that compound **B2** could reduce the HAL-associated side effects.

***In Vivo* Antifungal Potency of Compound B2.** *In vitro* antifungal activity assay revealed that compound **B2** exhibited significant synergistic antifungal activity against azole-resistant *C. albicans* when combined with FLC. To further assess the *in vivo* antifungal potency of compound **B2**, an azole-resistant *C. albicans* infection mice model was established by injection with resistant *C. albicans* strains *via* tail vein. Compound **B2** (10 mg/kg) and FLC (0.8 mg/kg) were administered by intraperitoneal injection for 5 days consecutively after 24 h post infection. As shown in Figure 7, compared to the saline group, the kidney fungal burden of mice treated with compound **B2** (10 mg/kg) combined with FLC (0.8 mg/kg) had a significantly reduced $\log_{10}$ CFU/g value from 6.35 to 4.89 ($P < 0.001$), which was superior to that of FLC ($P < 0.001$). In contrast, when compound **B2** was administrated alone, a loss of the *in vivo* potency was observed. The result showed that the combination of **B2** and FLC possessed excellent *in vivo* antifungal potency against azole-resistant *C. albicans*.

CONCLUSIONS

In summary, a series of novel HAL derivatives was designed in order to develop synergistic enhancers of existing antifungal drugs to treat azole-resistant candidiasis. In particular, compound **B2** exhibited excellent *in vitro* and *in vivo* synergistic antifungal efficacy against azole-resistant *C. albicans* when combined with FLC. As a synergistic enhancer, compound **B2** acted by regulating virulence-associated genes and down-regulating the CYP51-coding gene *ERG11* and efflux pump gene *CDR1*. Taken together, this work expands the SAR and antifungal mechanisms of HAL derivatives and discovered lead compound **B2** as a potent synergistic enhancer

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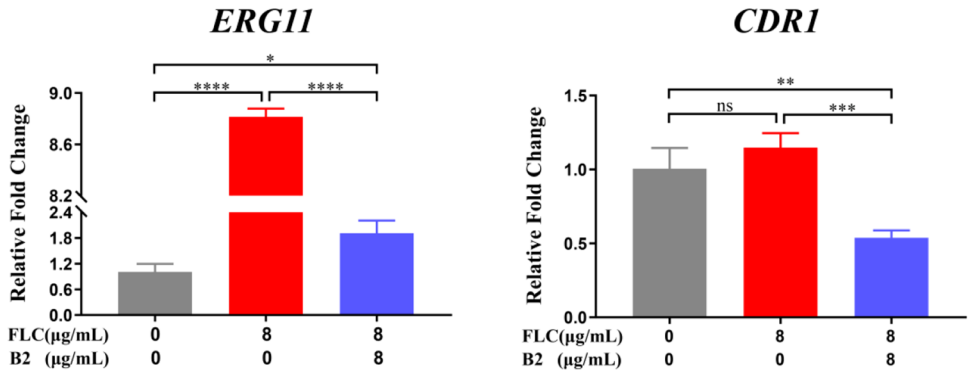

Figure 6. Regulatory effect of compound **B2** on *ERG11* and *CDR1* genes of azole-resistant *C. albicans* when used in combination with FLC through real-time RT-PCR analysis, with FLC as the control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, determined by Student's *t* test).

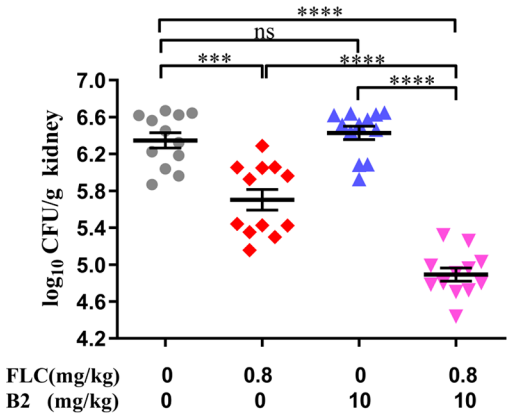

Figure 7. Kidney fungal burden of mice infected by azole-resistant *C. albicans* when treated with FLC, **B2**, or both in an azole-resistant *C. albicans* infection model (*** $P < 0.001$, **** $P < 0.0001$, determined by Student's *t* test).

to treat azole-resistant candidiasis. Further structural optimization and mechanism studies are currently in progress.

METHODS

Chemistry. *General Methods.* The used solvents and reagents were of analytical grade and were from commercial companies unless otherwise stated. Silica gel plates GF254 were used for thin-layer chromatography (TLC) analysis, which was purchased from Qingdao Haiyang Chemical (Qingdao, China). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE300 or AVANCE600 spectrometer. TMS was used as the internal standard. The chemical shifts (δ values) were expressed by parts per million (ppm). The coupling constants (J values) were expressed by hertz (Hz). The mass spectra or high-resolution mass spectra (HRMS) were recorded on an Agilent ultra-performance liquid chromatography to quadrupole time-of-flight mass spectrometry (UPLC−QTOF/MS) mass spectrometer. Reversed-phase high-performance liquid chromatography (RP-HPLC, Agilent 1260) was used to measure the purity of the target compounds. The measurement of purity was carried out under the conditions of MeOH/0.1%TFA·H_2O (v/v, 8/2) with a 0.5 mL/min flow rate on a C18 column. The purity of all compounds was greater than 95%.

Compounds **A1** and **A5** were prepared by referring to our previously reported synthetic methods.[12]

*4-(4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl)-1-(2,3-dihydrobenzofuran-5-yl)butan-1-one (**A1**).* White solid (160 mg), yield: 44.9%. ^{1}H NMR (600 MHz, DMSO-d_6, TMS): δ 7.88 (s, 1H), 7.81 (d, J = 8.4 Hz, 1H), 7.41 (d, J = 8.4 Hz, 2H), 7.34 (d, J = 8.4 Hz, 2H), 6.86 (d, J = 8.4 Hz, 1H), 4.82 (s, 1H), 4.63 (t, J = 8.7 Hz, 2H), 3.23 (t, J = 8.7 Hz, 2H), 2.91 (t, J = 6.9 Hz, 2H), 2.64−2.55 (m, 2H), 2.38−2.28 (m, 4H), 1.84−1.70 (m, 4H), 1.56−1.44 (m, 2H). ^{13}C NMR (151 MHz, DMSO-d_6, TMS): δ 198.55, 164.09, 149.70, 131.18, 130.86, 130.08, 128.46, 128.13 (s, 2C), 127.24 (s, 2C), 125.80, 109.11, 72.50, 70.01, 57.83, 49.40 (s, 2C), 38.31 (s, 2C), 35.89, 28.91, 22.55. HRMS (ESI) m/z: calcd for $C_{23}H_{26}ClNO_3$ $[M + H]^+$ 400.1674, found 400.1689.

*3-(4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl)-1-(6,7,8,9-tetrahydro-5H-benzo[7]annulen-2-yl)propan-1-one (**A5**).* White solid (53 mg), yield: 43.1%. ^{1}H NMR (600 MHz, $CDCl_3$, TMS): δ 7.71 (d, J = 1.7 Hz, 1H), 7.69 (dd, J = 7.7, 1.9 Hz, 1H), 7.46−7.42 (m, 2H), 7.32−7.29 (m, 2H), 7.18 (d, J = 7.7 Hz, 1H), 3.20 (t, J = 7.4 Hz, 2H), 2.91 (t, J = 7.4 Hz, 2H), 2.88−2.81 (m, 6H), 2.62−2.51 (m, 2H), 2.18−2.08 (m, 2H), 1.89−1.70 (m, 5H), 1.69−1.59 (m, 4H). ^{13}C NMR (151 MHz, $CDCl_3$, TMS): δ 198.98, 149.50, 146.85, 143.88, 134.92, 132.82, 129.30, 128.57, 128.43 (s, 2C), 126.11 (s, 3C), 70.90, 53.36, 49.45 (s, 2C), 38.38 (s, 2C), 36.67, 36.61, 36.16, 32.53, 28.09, 27.93. HRMS (ESI) m/z: calcd for $C_{25}H_{30}ClNO_2$ $[M + H]^+$ 412.2038, found 412.2054.

*4-(4-Chlorophenyl)-1-(4-hydroxy-4-(6,7,8,9-tetrahydro-5H-benzo[7]annulen-2-yl)butyl)piperidin-4-ol (**B2**).* Compound **14b** (72 mg, 0.169 mmol, 1.0 equiv) and $NaBH_4$ (32 mg, 0.845 mmol, 5.0 equiv) were dissolved in 20 mL of anhydrous MeOH, and the mixture was stirred at room temperature (25 °C) for 1.5 h. Following this, the reaction solvent was removed by concentrated *in vacuo* reduced pressure. Then, 25 mL of water (repeated twice) was added to dilute and wash the reaction solution, which was further extracted with 25 mL of EtOAc (repeated twice). The organic layer solution was combined and washed with saturated 25 mL of NaCl solution (repeated twice). Then, the reaction solution was dried with anhydrous Na_2SO_4. The crude product was obtained by concentrated *in vacuo* reduced pressure after drying and removing water, which was further purified by silica gel column chromatography (DCM/MeOH = 20/1, v/v) to afford compound **B2** (59 mg, 81.7% yield) as a white solid. ^{1}H NMR (600 MHz, DMSO-d_6, TMS): δ 7.48 (d, J = 8.5 Hz, 2H), 7.36 (d, J = 8.5 Hz, 2H), 7.05 (s, 1H), 7.04−6.98 (m, 2H), 5.41 (s, 1H), 4.96 (s, 1H), 4.45 (s, 1H), 2.85−2.59 (m, 6H), 2.49−2.14 (m, 4H), 2.02−1.84 (m, 2H), 1.81−1.74 (m,

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2H), 1.66−1.40 (m, 10H). ^{13}C NMR (151 MHz, DMSO-d_6, TMS): δ 148.89, 144.02, 142.54, 141.27, 131.03, 128.58, 127.92 (s, 2C), 126.94 (s, 2C), 126.60, 123.40, 72.20, 69.38, 57.81, 49.04, 48.90, 37.48 (s, 2C), 37.29, 36.19, 35.66, 32.29, 28.31, 28.25, 22.82. HRMS (ESI) m/z: calcd for $C_{26}H_{34}ClNO_2$ $[M + H]^+$ 428.2351, found 428.2380. HPLC purity: 99.5%. Retention time: 7.286 min, eluted with MeOH/0.1%TFA·H_2O (v/v, 8/2).

***In Vitro* Antifungal Potency Test.** *In vitro* antifungal activity of the compounds was evaluated by determining the MIC_{80}. The test was performed using the microliquid dilution method by referring to CLSI (M27-A3).[35] Fungal cells were incubated to the exponential growth stage in YEPD medium and then collected and diluted with RPMI 1640 medium to 1 × 10^3 CFU/mL. The different concentration solutions of compounds were obtained by 2-fold serial dilutions in 96-well plates. The fungal cells were incubated at 35 °C for 48 h, except that *C. neoformans* H99 was incubated at 35 °C for 72 h. Then, the optical density at 630 nm (OD_{630}) was measured with a spectrophotometer to calculate the inhibition rate of each well, which was used to evaluate the antifungal activity of the compounds. Each compound was tested in triplicate.

***In Vitro* Synergistic Antifungal Activity Test.** The test was performed using the checkerboard microdilution method by referring to CLSI (M27-A3).[35] Fungal cells were incubated to the exponential growth stage in YEPD medium, then collected and diluted with RPMI 1640 medium to 1 × 10^3 CFU/mL. Fungal cells containing the tested compounds with different concentrations were added to 24-well plates for the preparation of fungal suspension and then transferred to 96-well plates. Then, FLC was added into the 96-well plates and serially double-diluted. The azole-resistant *C. albicans* cells were static cultured at 35 °C for 48 h. Then, the OD_{630} with a spectrophotometer was measured to calculate the inhibition rate of each well, which was analyzed referring to Loewe additivity theory and expressed by the FICI.[36] FICI < 0.5 indicates synergistic effect, FICI > 4 indicates antagonistic effect, and 0.5 ≤ FICI ≤ 4 indicates indifferent.[35] Each compound was tested in triplicate.

Time-Growth Curve Assay. The assay was modified with reference to the previously reported protocol.[12,13] Azole-resistant *C. albicans* cells were incubated to the exponential growth stage in YEPD medium and diluted with RPMI 1640 medium to 1 × 10^6 CFU/mL. Then, FLC and compound **B2** or both were added into 5 mL of fungal suspension as drug groups, and the cells without any treatment were used as the control group. The azole-resistant *C. albicans* cells were shaking-cultured (200 rpm/min) at 30 °C, and the OD_{630} with a spectrophotometer was measured to calculate the numbers of azole-resistant *C. albicans* cells at different specified times. This assay was performed in triplicate.

Filamentation Assay. The assay was modified with reference to the previously reported protocol.[37] Azole-resistant *C. albicans* cells were incubated to the exponential growth stage in YEPD medium and diluted with spider medium to 1 × 10^6 CFU/mL. The fungal suspension was transferred to a 24-well plate with 2 mL per well. Then, FLC and compound **B2** or both were added to the 24-well plate as drug groups, and the cells without any treatment were used as the control group. The azole-resistant *C. albicans* cells were static cultured at 37 °C for 3 h, and then, the fungal morphological differences were recorded on a live cell imaging inverted microscope.

Biofilm Formation Assay. The assay was modified with reference to the previously reported protocol.[37,38] Azole-resistant *C. albicans* cells were incubated to the exponential growth stage in YEPD medium and diluted with RPMI 1640 medium to 1 × 10^6 CFU/mL. The fungal suspension was added to 96-well plates with 100 μL per well. The azole-resistant *C. albicans* were static cultured at 37 °C for 1.5 h. Then, the cells were washed three times with 150 μL of phosphate buffer saline (PBS) and the nonadherent cells were removed. Drugs including FLC and compound **B2** or both were added to each well, and then, the azole-resistant *C. albicans* cells were further static cultured at 37 °C for 24 h. After that, the upper RPMI 1640 medium liquid was absorbed, removed, and gently washed three times with 150 μL of PBS. Then, 120 μL of XTT/menadione solution was added to the 96-well plates, and the cells were further static cultured at 37 °C for 3 h, avoiding light. Then, 80 μL of supernatant was absorbed from each well and transferred to a 96-well blank plate. The OD_{492} value of each well was measured by a spectrophotometer. Finally, the XTT reduction assay was used to calculate the semiquantitative measurement of biofilm formation rate, and the changes of XTT staining were recorded by photos at the same time.

***In Vivo* Antifungal Potency.** The assay was performed according to the previously reported protocol.[12] Female mice (breed, ICR; weight, 18−22 g; age, 4−6 weeks) were used to build the *in vivo* experimental model, which were from the Shanghai Experimental Animal Center, Chinese Academy of Sciences. The drug solutions were prepared in advance and divided into four groups: control group (normal saline, NS), FLC group (0.8 mg/kg, in NS), compound **B2** group (10 mg/kg, suspended in NS with 1.5% glycerin and 0.5% Tween 80), and the coadministration group of FLC and compound **B2**. All mice were intraperitoneal injected with 0.2 mL of cyclophosphamide (100 mg/kg, in NS) before the inoculation, which was used as an immunosuppressive agent to destroy the immune system of mice. After 24 h, each mouse was inoculated with a 0.2 mL inoculum of azole-resistant *C. albicans* (1 × 10^6 CFU/mL) *via* a tail vein. The mice were administered with drug solutions by intraperitoneal injection once a day at regular times. After continuous administration for 5 days, all the mice were sacrificed, and then, they were dissected and their kidneys were removed and weighed. Following this, the left kidneys were put into centrifuge tubes; to each tube was added 1 mL of NS. The kidney tissue homogenate was obtained through a high-speed tissue homogenizer and further diluted with NS. The diluent kidney tissue homogenates were spread on Sabouraud dextrose agar (SDA) medium plates with chloramphenicol (100 μg/mL) by an inoculating loop and static cultured at 35 °C for 48 h. Finally, the number of single colonies on each SDA medium were counted, and the fungal burden of the kidney tissue was further calculated. ANOVA was used to analyze the differences among the groups.

Microscopic Observation of Fungal Morphology. Microscopic morphology of azole-resistant *C. albicans* cells was observed by TEM, which was a reference to the reported protocol with some modifications.[12] Azole-resistant *C. albicans* cells were incubated to the exponential growth stage in YEPD medium and diluted with RPMI 1640 medium to 5 × 10^5 CFU/mL. Then, the drugs were added including FLC, compound **B2**, or both into 10 mL of fungal suspension, and the control group was treated without drugs. The cells of each group were incubated with constant shaking (200 rpm/min) at

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30 °C for 8 h. Then, they were washed with PBS three times, and 800 μL of fixative solution (4% paraformaldehyde) was added and fixed at 4 °C overnight. Then, the cells were washed with NS and fixed with 1% phosphotungstic acid for 1.5 h. After being treated with dehydration, embedding, and sectioning, they were observed and photographed under a transmission electron microscope.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.0c00849.

Discussions of strains, culture, and agents used, mature biofilm destruction assay, real-time RT-PCR analysis, HTRF assay, water solubility test, and chemical synthesis and structural characterization, figures of biofilm formation percentages compared to the untreated groups, dose response curves, ^{1}H and ^{13}C NMR spectra, ESI-HRMS spectra, and HPLC spectra, table of primers for real-time RT-PCR, and schemes of synthetic pathways (PDF)

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‖W.Y. and J.T. contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

IFIs, invasive fungal infections; *C. albicans*, *Candida albicans*; CYP51, lanosterol 14α-demethylase; FLC, fluconazole; HAL, haloperidol; FICI, fractional inhibitory concentration index; SAR, structure−activity relationships; THF, tetrahydrofuran; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DEAD, diethyl azodicarboxylate; *n*-BuLi, *n*-butyllithium; MIC_{80}, minimum inhibitory concentration with inhibition over 80%; VRC, voriconazole; ITC, itraconazole; *C. auris*, *Candida auris*; S_w, water solubility; *C. alb.*, *Candida albicans*; *C. gla.*, *Candida glabrata*; *C. par.*, *Candida parapsilosis*; *C. neo.*, *Cryptococcus neoformans*; XTT, 2,3-bis(2-hydroxyethylthio)naphthalene-1,4-dione; TEM, transmission electron microscopy; D2R, dopamine D2 receptor; cAMP, cyclic adenosine monophosphate; HTRF, homogeneous time-resolved fluorescence; CFU, colony-forming units; RIS, risperidone; TLC, thin-layer chromatography; ppm, parts per million; HRMS, high-resolution mass spectra; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; OD, optical density; PBS, phosphate buffer saline; NS, normal saline; SDA, Sabouraud dextrose agar

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