ACS Infectious Diseases

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ACS Infect. Dis., **Just Accepted Manuscript** • DOI: 10.1021/acsinfecdis.9b00197 • Publication Date (Web): 24 Sep 2019

Just Accepted

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Drug Repurposing of Haloperidol: Discovery of New Benzocyclane Derivatives as Potent Antifungal Agents against Cryptococcosis and Candidiasis

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Despite the high morbidity and mortality of invasive fungal infections (IFIs), effective and safe antifungal agents are rather limited. Starting from antifungal lead compound haloperidol that was identified by drug repurposing, a series of novel benzocyclane derivatives were designed, synthesized and assayed. Several compounds showed improved antifungal potency and broader antifungal spectrum. Particularly, compound **B10** showed good inhibitory activities against a variety of fungal pathogens, and was proven to be inhibitors of several virulence factors important for drug resistance. In the *in vivo* cryptococcosis and candidiasis models, compound **B10** could effectively reduce the brain fungal burden of *Cryptococcus neoformans*, and synergize with fluconazole to treat resistant *Candida albicans* infections. Preliminary antifungal mechanism studies revealed that compound **B10** regained cell membrane damage and down-regulated the overexpression of *ERG11* and *MDR1* genes when used in combination with fluconazole. Taken together, haloperidol derivative **B10** represent a promising lead compound for the development of new generation of antifungal agents.

KEYWORDS: Antifungal, haloperidol, drug repurposing, *Cryptococcus neoformans*, drug resistance, *Candida albicans*

It is estimated that there are approximately 5 million species of fungi widely prevalent in environment, out of which about 300 can cause human infections, but only 20-25 of them are common in clinic.[1] Invasive fungal infections (IFIs) are mostly caused by opportunistic pathogens and often occur in immunocompromised people, such as HIV-infected and organ transplantation patients or people undergoing chemotherapy.[2-6] Candida, Cryptococcus, Aspergillus and Pneumocystis are the most common fungal pathogens causing approximately 2 million infected people and 1 million deaths each year. [7-9]

With rapid expansion of the immunodeficiency population, the morbidity of IFIs increases each year, and the mortality remains high.[10-17] For example, Cryptococcus and Candida infect over 1,000,000 and 400,000 people each year, while the mortality can be up to 20-70% or 46-75%, respectively.[11] However, the development of antifungal drugs has lagged far behind. Since 1950s, there have been only four classes of antifungal agents (i.e. polyenes, azoles, echinocandins and flucytosine) are available for the treatment of IFIs.[18, 19] Even though, they can hardly achieve a fast action and significant efficacy against severe IFIs such as cryptococcal meningitis (CM).[20-22] In some cases, drug treatment has to last for several months or even longer.[23] Meanwhile, most antifungal agents have moderate to severe side effects, especially polyenes.[24-28] Although echinocandins are well tolerated by patients, they are poorly effective against Cryptococcus.[29, 30] Moreover, severe drug resistance has been developed due to broad use of antifungal agents, especially that the resistance of

Candida albicans towards azoles is becoming increasingly common.[31-33] Therefore, the development of new generation of antifungal agent is highly desirable.

Recently, drug repurposing (also called drug repositioning) has been proven to be an promising approach to accelerate drug development not only in identifying new uses for old drugs but also offering new lead compounds.[34] A series of "non-antifungal" drugs with antifungal activity were identified by screening libraries of marketed drugs.[29] Among them, haloperidol, a butylbenzoic antipsychotics drug, was reported to exhibit inhibitory effect against *Candida albicans*[35] and a synergistic effect against Malassezia[36]. More recently, Garneau-Tsodikova *et al*. reported bromperidol derivatives had synergistic effects with triazole antifungal agents to treat *Candida* infections.[37] Thus, haloperidol represents a good lead compound for the development of novel antifungal agents. However, the structure-activity relationship (SAR) of haloperidol is still unknown, and further improving the antifungal potency and clarifying the antifungal mechanisms remains a highly challenging task.

Herein, a series of haloperidol derivatives were designed and synthesized using the scaffold hopping approach (**Figure 1**). As compared with haloperidol, several benzocyclane derivatives showed improved antifungal activity and broader antifungal spectrum. In particular, compound **B10** exhibited potent *in vitro* and *in vivo* antifungal activity in treating cryptococcosis and candidiasis. Interestingly, compound **B10** revealed excellent synergistic effects with fluconazole (FLC) against FLC-resistant *C. albicans*. The synergistic mechanisms were preliminarily clarified by transmission

electron microscopy and real-time RT-PCR analysis. Thus, compound **B10** represents a promising lead compound for the development of novel antifungal agents.

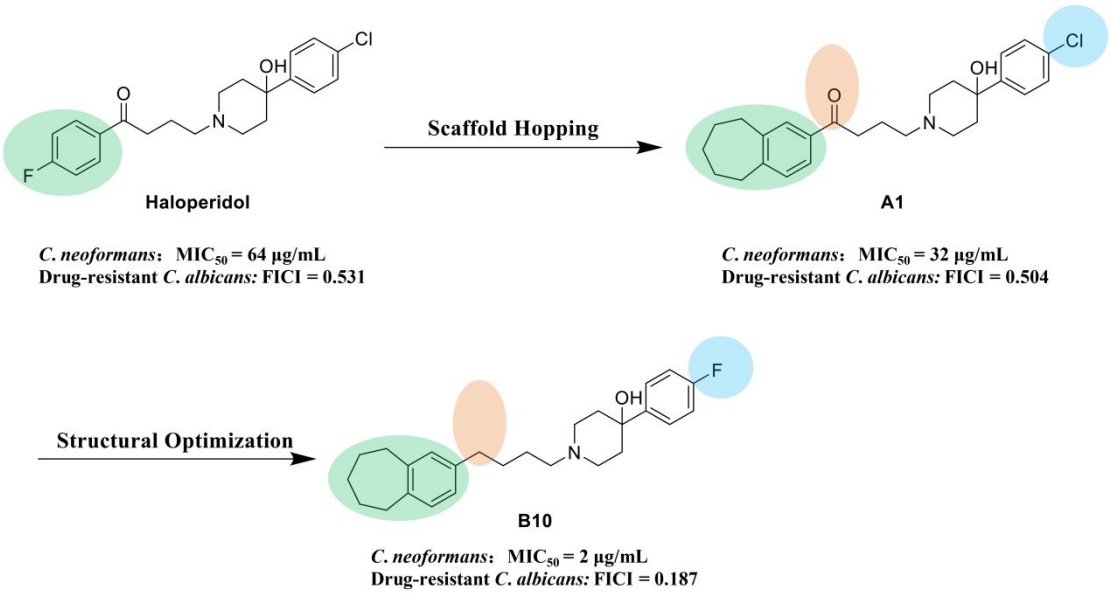


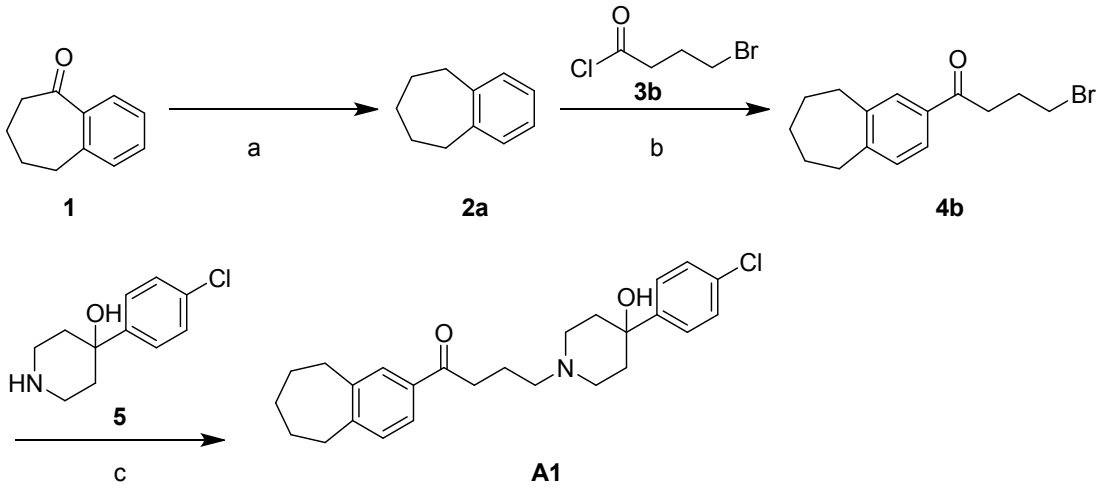
Figure 1. Structural optimization process of antifungal lead compound haloperidol.

RESULTS AND DISCUSSION

Chemistry. Chemical synthesis of benzocyclane derivatives are described in **Schemes 1-5**. Starting from compound **1**, its carbonyl group was reduced by Et_3SiH and trifluoroacetic acid (TFA), followed by Friedel-Crafts acylation with 4-bromobutanoyl chloride (**3b**) to afford intermediate **4b**, which was further substituted with 4-(4-chlorophenyl)piperidin-4-ol (**5**) to give target compound **A1** (**Scheme 1**). Ester **7** was obtained by Friedel-Crafts acylation of intermediate **2** with methyl 4-chloro-4-oxobutanoate (**4**), and then hydrolyzed to acid **8**, which was treated with intermediate **5** to afford target compound **A2** (**Scheme 2**). After the reduction of intermediate **7**, the resulting compound **9** was hydrolyzed into acid **10**, and then condensed with intermediate **5** in the presence of HATU and Et_3N to afford target

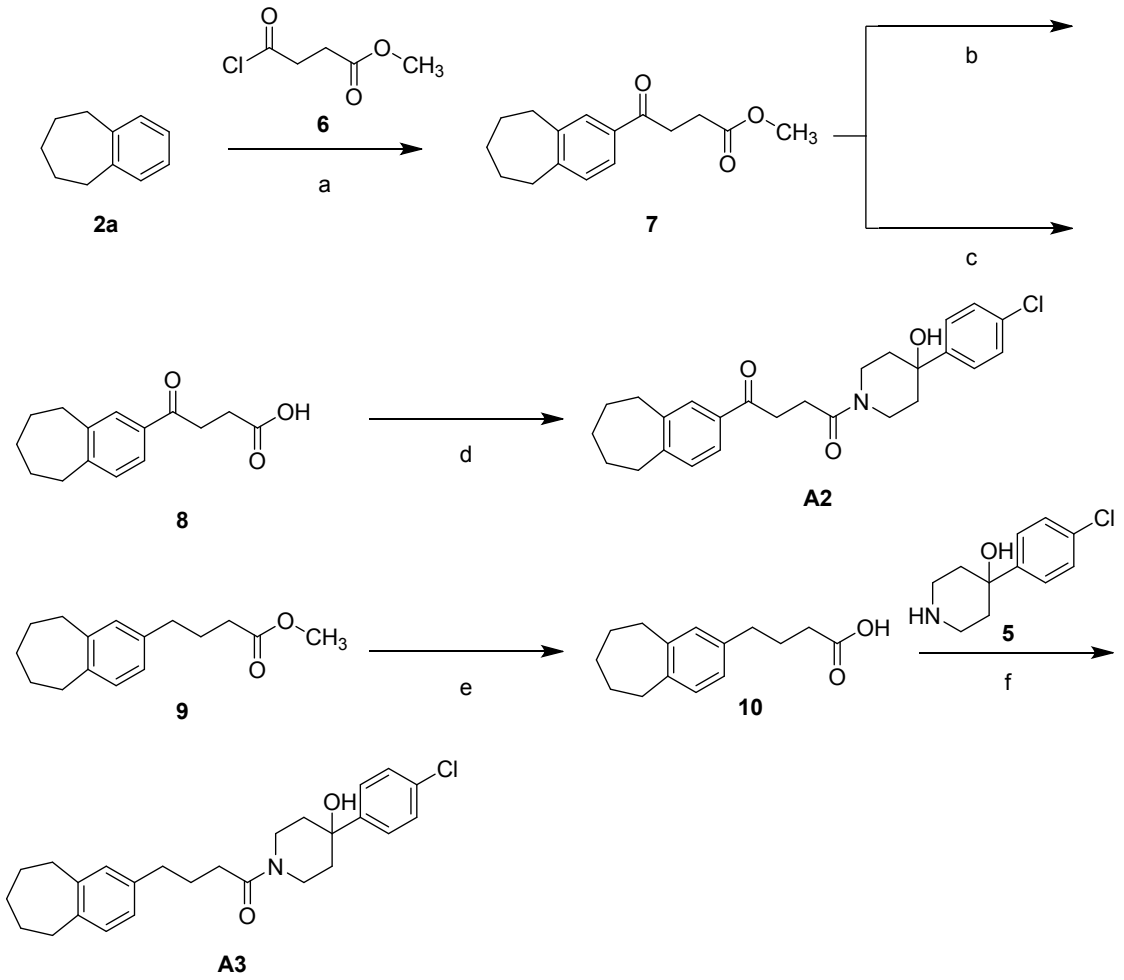
compound **A3** (**Scheme 2**). By a similar procedure depicted in **Schemes 1** and **2**, intermediates **11a-11e** were prepared from compounds **2a-2c** via acylation and reduction reaction, and then were substituted with intermediate **5** to afford target compounds **B1**-**B7** (**Scheme 3**). Intermediate **3b** was substituted by various piperazine or piperidine derivatives (**12a-12f**) to give target compounds **B8-B14** (**Scheme 4**). As shown in **Scheme 5**, compounds **13a-13d** were treated with compound **14** in the presence of n-BuLi and THF to afford intermediates **15a-15d**, then the benzyl group was removed by hydrogenation, and finally reacted with intermediate **3b** to afford target compounds **B15-B18**.

Scheme 1[a]



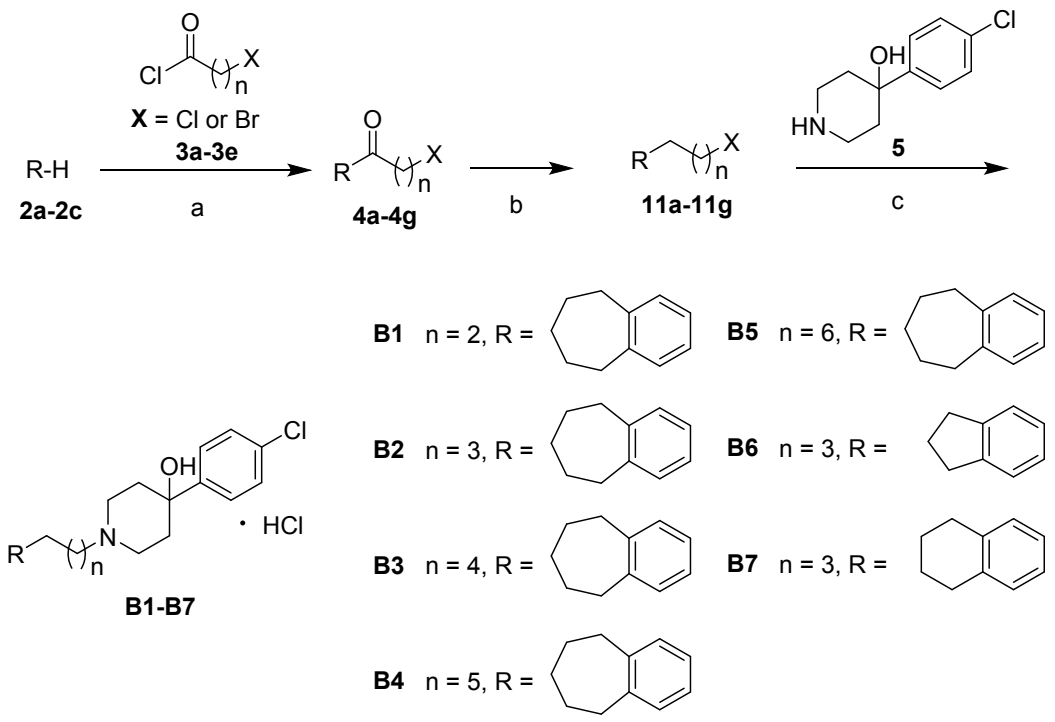
[a]**Reagents and conditions:** a) Et_3SiH, TFA, 60 °C, N_2, 4 h, yield 65.9%, b) $AlCl_3$, CH_2Cl_2, 0 °C, 1.5 h, yield 86.9%, c) DIPEA, DMF, 35 °C, 16 h, yield 45.1%.

Scheme 2[a]



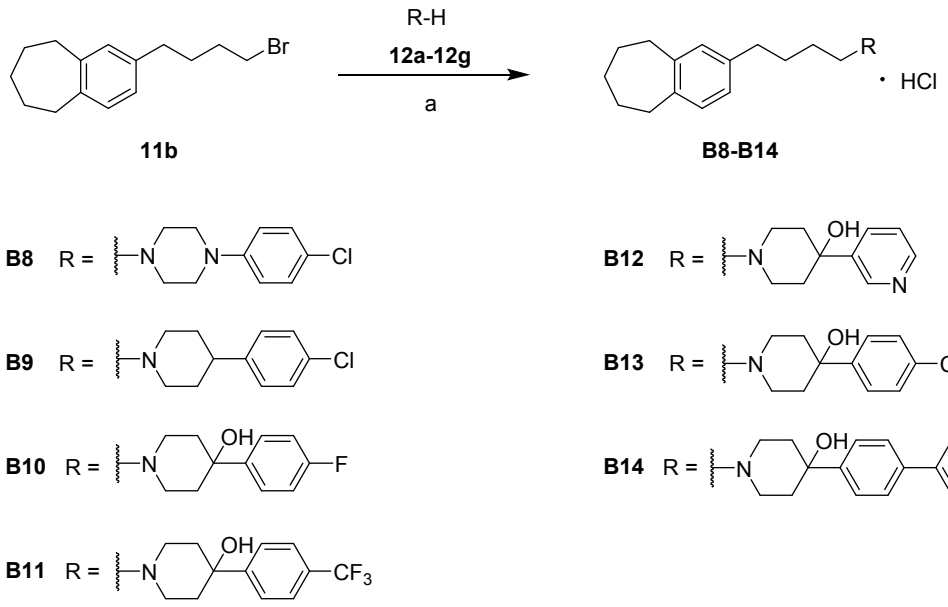
[a]**Reagents and conditions:** a) $AlCl_3$, CH_2Cl_2, 0 °C, 1.5 h, yield 69.1%, b) $LiOH \cdot H_2O$, THF/MeOH/H_2O, rt, 3 h, yield 84.4%, c) Et_3SiH, TFA, 60 °C, N_2, 4 h, yield 86.3%, d) HATU, Et_3N, DMF, rt 3 h, yield 80.1%, e) $LiOH \cdot H_2O$, THF/MeOH/H_2O, rt, 3 h, yield 79.6%, f) HATU, Et_3N, DMF, rt, 3 h, yield 79.8%.

Scheme 3[a]



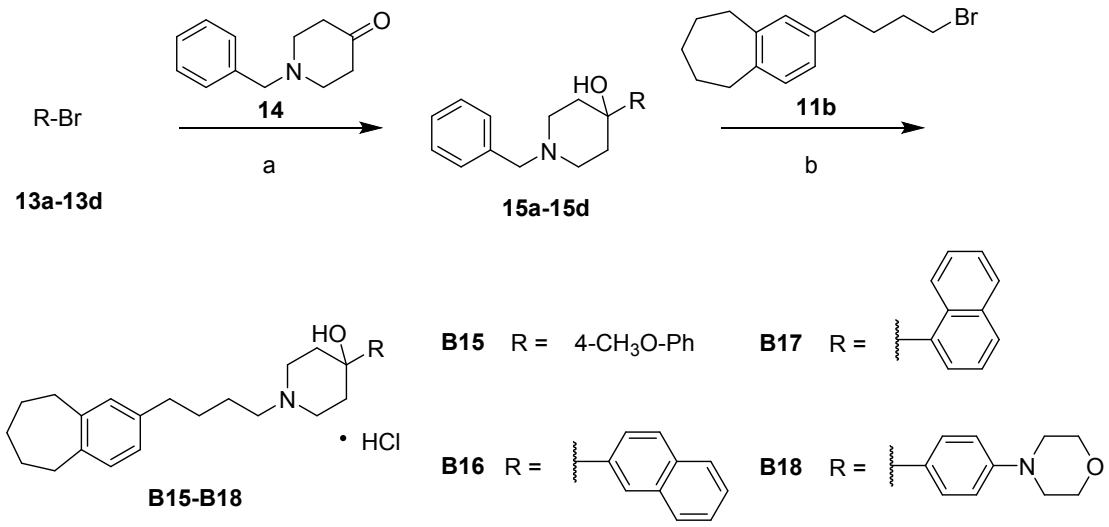
[a] **Reagents and conditions:** a) $AlCl_3$, CH_2Cl_2, 0 °C, 1.5 h, yield 78.9-86.8%, b) Et_3SiH, TFA, 60 °C, N_2, 4 h, yield 71.5-86.4%, c) ① DIPEA, DMF, 60 °C, 16 h ② NH_4Cl, yield 37.1- 53.4 %.

Scheme 4[a]



[a] **Reagents and conditions:** a) DIPEA, DMF, 60 °C, 16 h, yield 30.7-50.9%.

Scheme 5[a]



[a] **Reagents and conditions:** a) n-BuLi, THF, -78 °C-rt, N_2, overnight, yield 48.1-55.4%, b) ① H_2/Pa(OH)$_2$-C, MeOH, 30 °C, 6 h, ② DIPEA, DMF, 60 °C, 16 h, yield 40.9-49.8%.

Antifungal Susceptibility Testing. The *in vitro* antifungal activity of the target compounds were assayed against *C. albicans* SC5314, *C. glabrata* 8535, *C. neoformans* H99 *and C. neoformans* ATCC34877 using the plate microdilution method according to CLSI (M27-A)[38]. Most of the compounds showed moderate to excellent *in vitro* activities (**Table 1**). As compared with haloperidol, the replacement of the fluorophenyl group with the 6,7,8,9-tetrahydro-5*H*-benzo[7]annulene scaffold (compound **A1**) led to slight increase of the antifungal activity. When compound **A1** was added an additional carbonyl group in the linker, the antifungal activity was lost (compounds **A2** and **A3**). In contrast, compound **B2** showed much better activity than compound **A1** when the carbonyl group was removed. Variation of the linker length had significant impact on the antifungal activity and antifungal spectrum. For example,

among compounds **B1-B5**, compound **B2** with a linker length of 4 carbon atoms showed the best activity against *C. neoformans* (MIC_{50} = 8 μg/mL), while compound **B3** (linker length: 5 carbon atoms) exhibited the best activity against *C. albicans* (MIC_{50} = 16 μg/mL) and compound **B1** (linker length: 3 carbon atoms) exhibited the best activity against *C. glabrata* (MIC_{50} = 4 μg/mL). In contrast, the change of the cycloalkane part had little effect on the antifungal activities because compounds **B6** and **B7** showed similar inhibitory activity against *C. albicans*, *C. glabrata* and *C. neoformans* to compound **B2**. The replacement of the piperidine group to piperazine (compound **B8**) led to loss of the antifungal activity. The removal of hydroxyl group had little effect on the anti-*Cryptococcus* activity, whereas the anti-*Candida* potency was decreased for compound **B9**.

The substituent groups on the terminal benzene ring were important for the antifungal activity. Generally, the small and electron-withdrawing substituent groups were favorable for the antifungal activity. 4-Fluoro derivative **B10** showed the best activity, whereas 4-methyl (**B13**) and 4-methoxyl (**B15**) derivatives were less potent. The benzene/pyridine exchange was tolerated because compound **B12** also showed good activity. When the phenyl group was replaced or extended by more steric groups (*e.g*. naphthyl, biphenyl), obvious decrease of the antifungal activity was observed.

Table 1. *In Vitro* Antifungal Activity of Compounds against *C. albicans* SC5314, *C. glabrata* 8535, *C. neoformans* H99 and *C. neoformans* ATCC34877 (MIC_{50}, μg/mL).

Cmpd.	*C. alb.* SC5314	*C. gla.* 8535	*C.neo.* H99	*C.neo.* ATCC 34877	Cmpd.	*C. alb.* SC5314	*C. gla.* 8535	*C.neo.* H99	*C.neo.* ATCC 34877
A1	128	64	32	32	**B10**	16	4	2	2
A2	>128	128	>128	128	**B11**	16	4	4	4
A3	>128	>128	>128	128	**B12**	64	8	2	2
B1	32	4	16	8	**B13**	>128	16	8	8
B2	32	8	8	4	**B14**	>128	>128	8	16
B3	16	8	16	8	**B15**	64	8	16	16
B4	64	8	64	16	**B16**	>128	>128	16	16
B5	64	2	8	16	**B17**	32	8	8	8
B6	64	16	8	4	**B18**	128	>128	64	32
B7	32	16	8	8	**HAL**[a]	>128	64	64	32
B8	>128	>128	>128	>128	**FLC**[a]	0.25	4	4	4
B9	>128	32	4	16					

[a]Abbreviations: FLC, Fluconazole, HAL, Haloperidol, *C. alb.*, *Candida albicans*, *C. gla.*, *Candida glabrata*, *C. neo.*, *Cryptococcus neoformans*.

The Evaluation of Synergistic Inhibitory Effects against FLC-Resistant *C. albicans*. The synergistic antifungal activity of benzocyclane derivatives against FLC-resistant *C. albicans* clinical isolates (strains number: 0304103, 4108, 7781) were also investigated. The synergistic effects were evaluated by the fractional inhibitory concentration (FIC) index.[39] Most of the target compounds exhibited excellent synergistic inhibitory effect with *C. albicans*, with the FIC index (FICI) ranging from 0.125 to 0.312. Considering the antifungal activity and synergistic effects, compounds **B10** and **B12** were selected for further evaluations. In *vitro* inhibitory activities against other *Candida* spp., including *Candida tropicalis*, *Candida parapsilosis* and *Candida krusei* were assayed (**Table 3**). The results indicated that they only showed moderate inhibitory activity agianst these strains, and the activity of compound **B10** was better than that of compound **B12**.

Table 2. Antifungal Activity and Synergistic Effects of Target Compounds with FLC against FLC-Resistant *C. albicans.*

Strains	0304103 (MIC_{50}, μg/mL)					4108 (MIC_{50}, μg/mL)					7781 (MIC_{50}, μg/mL)				
	used alone		used in combination		FIC Index[a]	used alone		used in combination		FIC Index[a]	used alone		used in combination		FIC Index[a]
	FLC	Cmpd.	FLC	Cmpd.		FLC	Cmpd.	FLC	Cmpd.		FLC	Cmpd.	FLC	Cmpd.	
A1	> 64	> 64	2	32	0.504	> 64	> 64	2	16	0.281	> 64	64	2	16	0.281
A2	> 64	> 64	> 64	> 64	>2	> 64	> 64	> 64	> 64	> 2	> 64	> 64	> 64	> 64	>2
A3	> 64	> 64	> 64	> 64	>2	> 64	> 64	> 64	> 64	>2	> 64	> 64	> 64	> 64	>2
B1	> 64	> 64	2	16	0.281	> 64	64	1	16	0.266	> 64	> 64	8	8	0.25
B2	> 64	32	4	8	0.312	> 64	64	2	8	0.156	> 64	> 64	2	8	0.156
B3	> 64	16	2	8	0.531	> 64	64	4	8	0.187	> 64	> 64	8	8	0.250
B4	> 64	64	1	64	1.01	> 64	> 64	4	16	0.312	> 64	> 64	0.5	16	0.258
B5	> 64	16	1	16	1.02	> 64	> 64	8	8	0.25	> 64	64	0.25	32	0.504
B6	> 64	32	2	4	0.156	> 64	64	4	8	0.187	> 64	> 64	4	8	0.187
B7	> 64	32	4	8	0.312	> 64	64	4	16	0.312	> 64	> 64	4	8	0.187
B8	> 64	> 64	> 64	> 64	>2	> 64	> 64	> 64	> 64	>2	> 64	> 64	> 64	> 64	>2
B9	> 64	> 64	4	16	0.312	> 64	> 64	4	8	0.187	> 64	> 64	4	8	0.187
B10	> 64	32	4	4	0.187	> 64	64	4	4	0.125	> 64	32	4	4	0.187
B11	> 64	64	4	8	0.187	> 64	64	4	8	0.187	> 64	64	8	8	0.250
B12	> 64	> 64	2	8	0.156	> 64	> 64	2	8	0.156	> 64	> 64	2	8	0.156

B13	> 64	32	4	4	0.187	> 64	> 64	4	8	0.187	> 64	> 64	2	16	0.281
B14	> 64	> 64	2	8	0.156	> 64	> 64	1	8	0.141	> 64	> 64	2	8	0.156
B15	> 64	64	8	8	0.250	> 64	> 64	2	16	0.281	> 64	> 64	2	16	0.281
B16	> 64	64	4	4	0.125	> 64	> 64	2	16	0.281	> 64	> 64	8	8	0.250
B17	> 64	16	4	8	0.562	> 64	> 64	2	32	0.531	> 64	> 64	1	32	0.516
B18	> 64	> 64	2	8	0.156	> 64	> 64	4	8	0.187	> 64	> 64	2	8	0.187
HAL	> 64	> 64	2	32	0.531	> 64	> 64	2	16	0.281	> 64	> 64	1	32	0.504

[a]FIC index is defined as the sum of MIC of each drug used in combination divided by the MIC of the drug used alone.[39] FICI < 0.5 = synergism, FICI ≥ 4 = antagonism, 0.5 ≤ FICI > 4 = indifferent.[39]

Table 3. In vitro antifungal activity of Haloperidol, compound B10 and B12 [a]

Cmpd.	*C. tro.* 3890	*C. par.* 20090	*C. kru.* 4996
B10	32	32	32
B12	32	64	64
HAL [a]	64	>128	64
FLC [a]	0.5	2	64

[a] Abbreviations: FLC, Fluconazole, HAL, Haloperidol, *C. tro*., *Candida tropicalis*, *C. par*., *Candida parapsilosis*, *C. kru*., *Candida krusei*.

Growth Curve Assay. The efficacy of compound **B10** against *C. neoformans* and FLC-resistant *C. albicans* were further investigated by growth curve assay. *C. neoformans* or *C. albicans* cells were treated with different concentrations of FLC

or/and compound **B10** for 48 h at 30 °C (**Figure 2**). It was found that compound **B10** could strongly and dose-dependently inhibited the growth of *C. neoformans*, and the effect is much stronger than that of FLC (**Figure 2A**). Compared with the untreated group, the $\log_{10}$CFU (colony-forming units) of cells treated with 4 μg/mL of compound **B10** was reduced from 7.82 to 6.96 at the terminal time 48 h. As shown in **Figure 2B**, when used in combination with FLC, compound **B10** could intensively inhibit the growth of FLC-resisitant *C. albicans*. Rather than FLC, the inhibitory effect was dependent on the concentration of compound **B10**. In particlular, when 32 μg/mL of compound **B10** was used in combination with FLC, the growth of fungi was almost completely inhibited (inhibition rate > 98%).

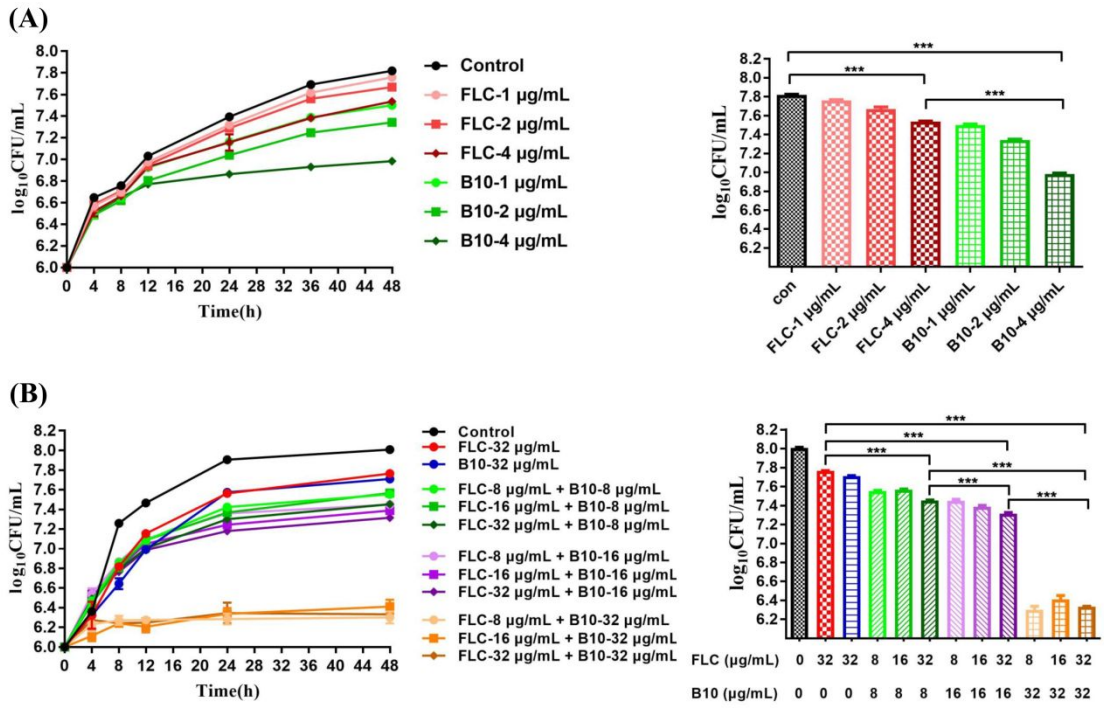


Figure 2. Growth curves assay of *C. neoformans* H99 and FLC-resistant *C. albicans* (strain number: 7781) treated with different concentrations of FLC or/and compound **B10**. (**A**) Growth curves of *C. neoformans* H99 cells treated with different

concentrations of FLC or compound **B10**, and the statistic comparison of different groups at 48 h. (**B**) Growth curves of *C. albicans* 7781 cells treated with different concentrations of FLC or/and compound **B10**, and the statistic comparison of different groups at 48 h. Statistical significance of groups was determined by Student's t test (*** $P < 0.001$).

The Inhibitory Activity of Compounds B10 and B12 on the Virulence Factors of *C. neoformans* and *C. albicans*. Virulence factors are produced by pathogens to enhance the pathogenicity or drug resistance, by improving the ability of colonization, invasiveness, immuno-evasion and immunosuppression, or the ability to resist antifungal drugs.[40] Targeting virulence factors is an attractive approach for the development of new generation of antifungal agents.[41, 42] Compound **B10** exhibited notable *in vitro* activity against *C. neoformans* and *C. albicans*. In order to further investigate the antifungal activity, the effect of compound **B10** against important virulence factors, such as melanin, urease, capsule, filamentation and biofilm were determined.

Melanin is an important virulence factor of Cryptococcus, which is mainly produced by phenoloxidase and located on the inner surface of cell wall.[43] It plays an important role in maintaining cell wall integrity, antioxidant, resistance to lysozyme and host immunity.[44-46] As shown in **Figure 3A**, compound **B10** significantly inhibited the production of melanin at the concentration of 32 μg/mL, while FLC and compound **B12** were inactive. Therefore, by inhibiting the production of melanin,

compound **B10** may kill Cryptococcus indirectly because the survival ability of Cryptococcus in the host was restricted.

Urease is a metalloenzyme catalyzing the hydrolysis of urea to ammonia and carbamate, and plays a critical role in the transfer of *C. neoformans* cells from blood into the brain.[47] As shown in **Figure 3B**, compound **B10** also inhibited the production of urease at the concentration of 32 μg/mL. Since that meningitis/encephalitis is a major lethal factor of *C. neoformans* infection, the inhibition of urease production may reduce the morbidity of cryptococcosis and improve the survival rate of the hosts.

As a major virulence factor of Cryptococcus, capsule plays a key role in the process of inducing phagocytosis, dissemination and immune escape.[48] Since the cupsule is primarily composed of glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GalXM), we evaluated the effect of compound **B10** on the production of these capsular polysaccharides. Compared with the untreated group, compound **B10** could significantly reduce the production of capsular polysaccharides of each gram wet fungal ($P < 0.05$, **Figure 3**C). As a result, compound **B10** might reduce the pathogenicity of *C. neoformans*.

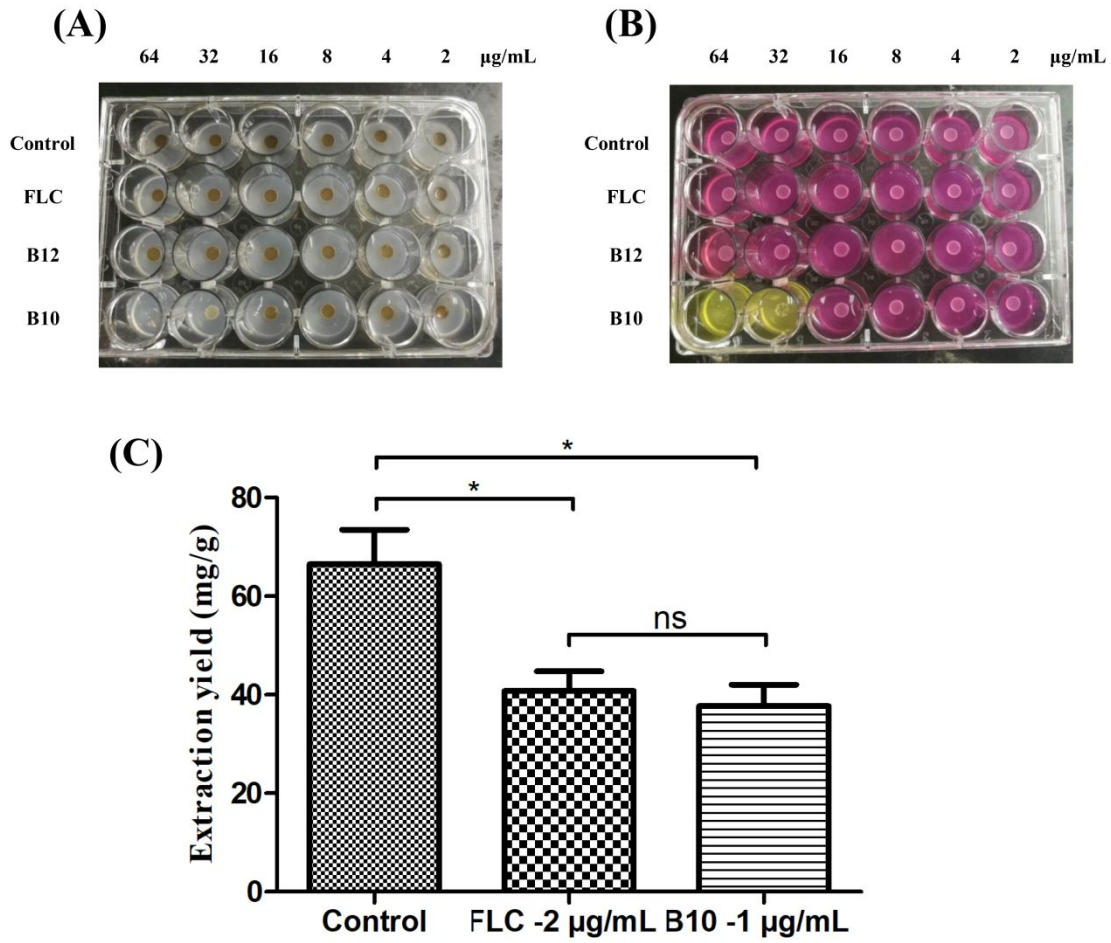


Figure 3. Inhibitory effects of compounds **B10**, **B12** and FLC on the production of melanin (**A**), urease (**B**) and capsular polysaccharides (**C**) of *C. neoformans* H99. Statistical significance of groups was determined by Student's t test (* $P < 0.05$).

Inhibitory Effect of Compound B10 on Filamentation of FLC-resisitent *C. albicans*. Filamentation is a key virulence factor of *C. albicans*. It was reported that hyphal *C. albicans* showed stronger ability of adhesion and invasion into the hosts than normal cells, and also was more likely to escape from the attack of host immune system.[49] The activity of compound **B10** on the yeast-to-hypha morphological transition of *C. albicans* was evaluated by culturing *C. albicans* in the RPMI 1640 medium (**Figure 4A**) and solid spider medium (**Figure 4B**, **Figure 4C**). The results

revealed that FLC couldn't inhibit the formation of hyphae at the concentration of 32 μg/mL, while compound **B10** was able to inhibit the morphological transition. When treated with 4 μg/mL of compound **B10**, the formation of hyphae was completely inhibited. As a result, the inhibition of filamentation by compound **B10** would restrain the pathogenicity of *C. albicans*.

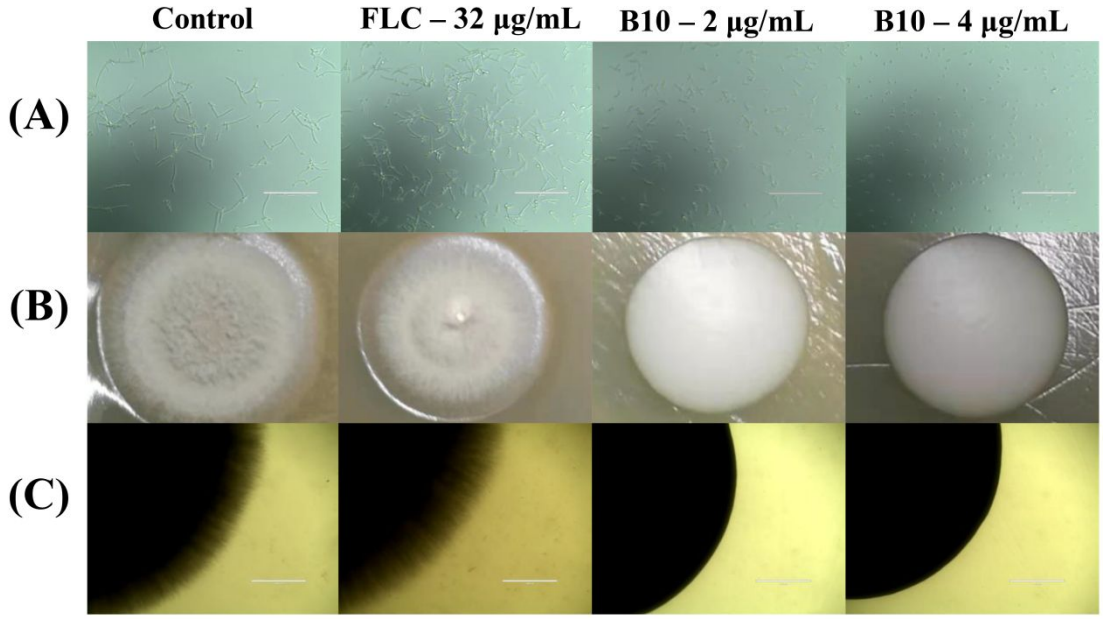


Figure 4. Inhibition of filamentation of *C. albicans* 7781. (**A**) Filamentation in liquid RPMI 1640 medium; (**B, C**) Filamentation in solid spider medium.

Inhibitory Activity of Compound B10 on the Biofilm Formation of *C. neoformans* H99 and FLC-resistant *C. albicans*. Fungal biofilm is a membrane-like multicellular complex attaching to a host cavity or a surface of a biological material, which is crucially important for the drug resistance to fungal pathogens.[50, 51] The effect of FLC and compound **B10** on the biofilm formation of *C. neoformans* and *C. albicans* was evaluated by the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-

tetrazolium-5-carboxanilide (XTT) reduction assay.[6] The results revealed that compound **B10** could significantly and dose-dependently inhibit the biofilm formation of both *C. neoformans* and *C. albicans* (**Figure 5**).

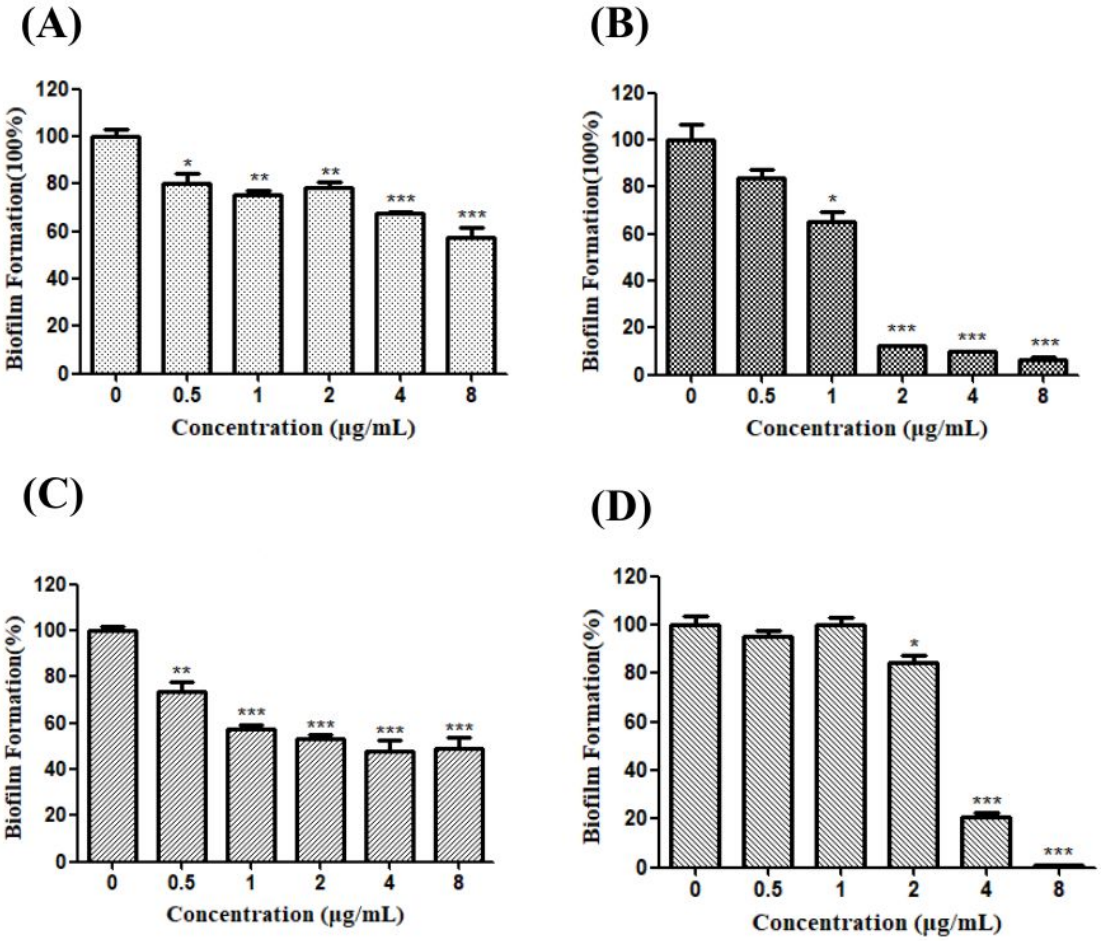


Figure 5. Inhibition of *C. neoformans* H99 biofilm formation by FLC (**A**) and compound **B10** (**B**) and *C. albicans* 7781 biofilm formation by FLC (**C**) and compound **B10** (**D**). Biofilm formation was evaluated by the XTT reduction assay, and the results were presented as a percentage in comparison with the control groups without compounds treatment. Biofilm formation results were represented as the mean ± standard deviation for three independent experiments. Statistical significance of groups was determined by Student's t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the control groups.

***In vivo* Efficacy Evaluation of Compound B10.** Prior to the *in vivo* studies, metabolic stability of compound **B10** was evaluated with mouse liver microsomes. As shown in **Table 4**, compound **B10** exhibited excellent metabolic stability with a half-live of 106.65 min and an intrinsic clearance rate of 51.17 mL/min/mg. Considering good *in vitro* activity and metabolic stability of compound **B10**, *in vivo* efficacy of compound **B10** towards *C. neoformans* and *C. albicans* was evaluated in a murine model of systemic cryptococcosis and candidiasis, respectively (**Figure 6A**). In the systemic cryptococcosis model, it was found that FLC and compound **B10** could reduce the fungal burden of the brain from $\log_{10}$ CFU/g value of 7.63 to 7.37 ($P < 0.001$) and 7.29 ($P < 0.001$), respectively. A similar protocol was followded in the systemic candidiasis model. Compared with the control group, compound **B10** had poor effect in reducing the fungal burden in the kidney when used alone at the dose of 10 mg/kg ($P > 0.05$). In contrast, FLC could effectively reduce the kidney fungal burden from $\log_{10}$ CFU/g value of 6.35 to 5.70 ($P < 0.001$). Interestingly, the combinational use of FLC and compound **B10** had synergistic *in vivo* antifungal effects, which significantly reduce the $\log_{10}$ CFU/g value to 4.76 ($P < 0.001$). The result revealed that compund **B10** could effectively inhibit *C. neoformans* and drug-resistant *C. albicans in vivo*, and showed potent therapeutic effects for the treatment of CM and candidiasis.

In order to further evaluate the *in vivo* efficacy of compound **B10**, we investigated the survival time of mice infected with *C. neoformans* and *C. albicans* when treated with the tested compounds (**Figure 6B**). In the CM model, the median survival time

(MST) of the mice treated with compound **B10** was longer than that of control group (8 days *vs* 6 days), but there was no significant difference ($P > 0.05$). As shown in the candidiasis model, the MST was significantly increased when treated with FLC (8 days *vs* 4 days, $P < 0.05$). And when FLC was used with compound **B10** in combination, the MST was significantly increased (10 days *vs* 4.0 days, $P < 0.001$), which was also longer than that in the FLC treated groups ($P < 0.05$).

The above results suggested that compund **B10** could effectively inhibit *C. neoformans* and drug-resistant *C. albicans in vivo*, and showed potent therapeutic effects for the treatment of CM and candidiasis. On the basis of the results from the *in vivo* models, we found that compound **B10** exhibited better antifungal potency in the candidiasis model than in the CM model, possibly due to its relatively poor ability to penatrate the blood brain barrier (**Table S1** in **Supporting Information**).

Table 4. Metabolic stability of compound B10 in mouse liver microsomes

	$T_{1/2}$[a] (min)	Cl_{int}[b] (mL/min/kg)
Ketanserin	21.29	256.36
B10	106.65	51.17

[a] Half-life. [b] Intrinsic Clearance.

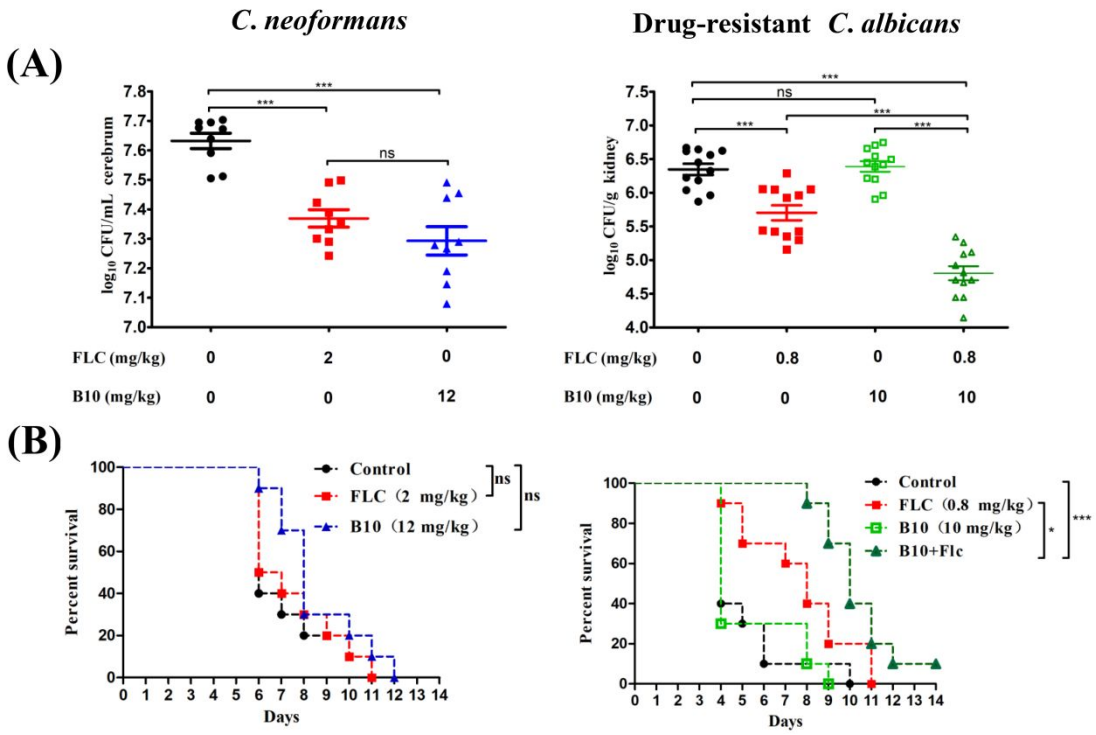


Figure 6. *In vivo* antifungal efficacy of compound **B10** and FLC in the CM and candidiasis model**.** (**A)** The fungal burden in the brain or kidney of infected mice treated with saline, FLC and/or compound **B10** in the CM or candidiasis model. (**B**) The survival time of mice treated with saline, FLC and/or compound **B10** in the CM and candidiasis model. Statistical significance among groups was determined by Log-rank (Mantel-Cox) Test. Data were represented as mean ± standard deviation (**P* < 0.05, *** *P* < 0.001).

Cell Cycle Analysis of *C. neoformans* H99 Treated with FLC and Compound B10. As shown above, compound **B10** exhibited potent *in vitro* and *in vivo* antifungal activities against *C. neoformans* and drug-resistant *C. albicans*. In order to understand how compound **B10** exerted the antifungal effects, the mechanisms of action was preliminary investigated. *C.neoformans* cells were treated with different

concentrations of FLC or compound **B10** for 24 h, the cell cycle was analyzed by flow cytometry. After being treated with FLC at 2 μg/mL and 4 μg/mL, the ratio of cells in the G1 phase were increased to 6.03% (**Figure 7B**) and 14.2% (**Figure 7C**), respectively, compared with the ratio of 3.74% in the control group (**Figure 7A**). When treated with 1 μg/mL and 2 μg/mL of compound **B10**, the ratio of cells in G1 phase were sharply increased to 83.7% (**Figure 7D**) and 87.5% (**Figure 7E**), respectively. As shown in **Figure 7F**, compound **B10** significantly arrested *C. neoformans* H99 cells in the G1 phase ($P < 0.001$). The results indicated that compound **B10** had similar cell cycle arrest effect to FLC, but its effect was much stronger than that of FLC.

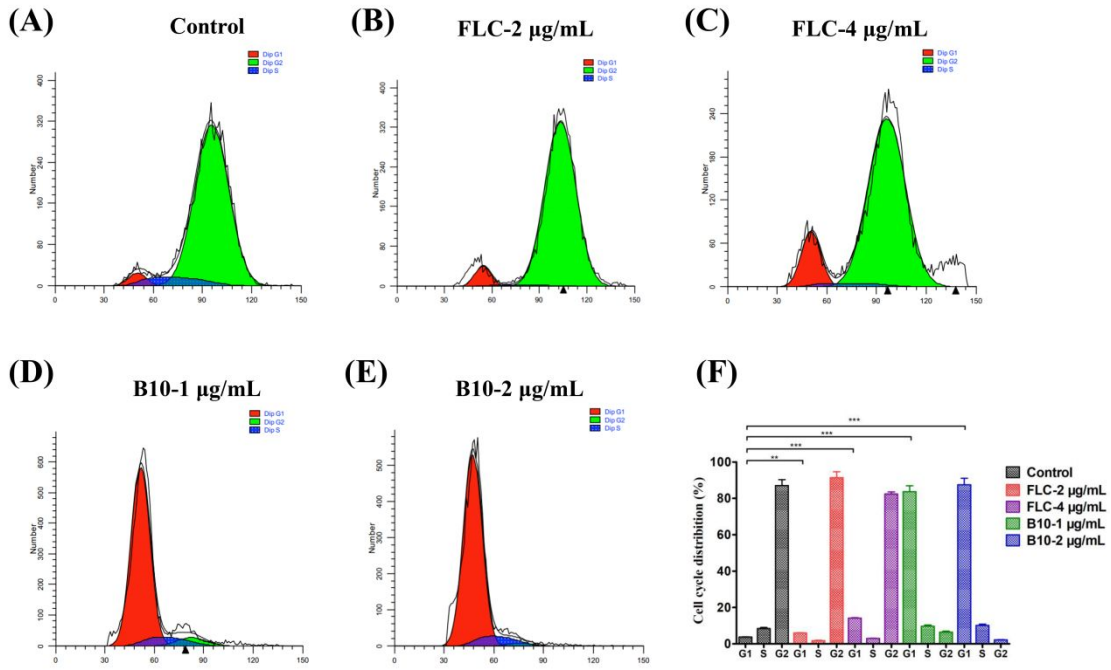


Figure 7. *C. neoformans* H99 cell cycle analysis by flow cytometry. (**A**) Untreated control group. (**B**) Cells treated with 2 μg/mL of FLC for 24 h. (**C**) Cells treated with 4 μg/mL of FLC for 24 h. (**D**) Cells treated with 1 μg/mL of **B10** for 24 h. (**E**) Cells treated with 2 μg/mL of **B10** for 24 h. (**F**) Mean percentages of cells in G1, S and G2.

The percentages in different phases were calculated by flow cytometry software. Statistical significance of groups was determined by Student's t test. Data were represented as mean ± standard deviation from three independent experiments. ** $P < 0.01$, *** $P < 0.001$ versus the control group.

Transmission Electron Microscopy Analysis. The morphological changes of *C. neoformans* cells after being treated with or without compound **B10** were examined with transmission electron microscopy (TEM). However, no obviously morphological structure changes were found when treated with 4 μg/mL of compound **B10** (**Figure 8A**). The morphological changes of *C. albicans* were also examined (**Figure 8B**). When treated with 16 μg/mL of FLC, the cell membrane of *C. albicans* was slightly damaged. When treated with compound **B10** and FLC in combination, the cells were remarkably damaged in which the cell membrane was totally destroyed and the cytoplasm leaked extensively. The results also revealed that compound **B10** could not damage FLC-resisitent *C. albicans* cells when used along. However, the cell membrane was severely damaged when it was used in combination with FLC, leading to the death of cells. Thus, the activity of FLC against the cell membrane of drug-resistant fungi might be regained by the synergistic effects of compound **B10**.

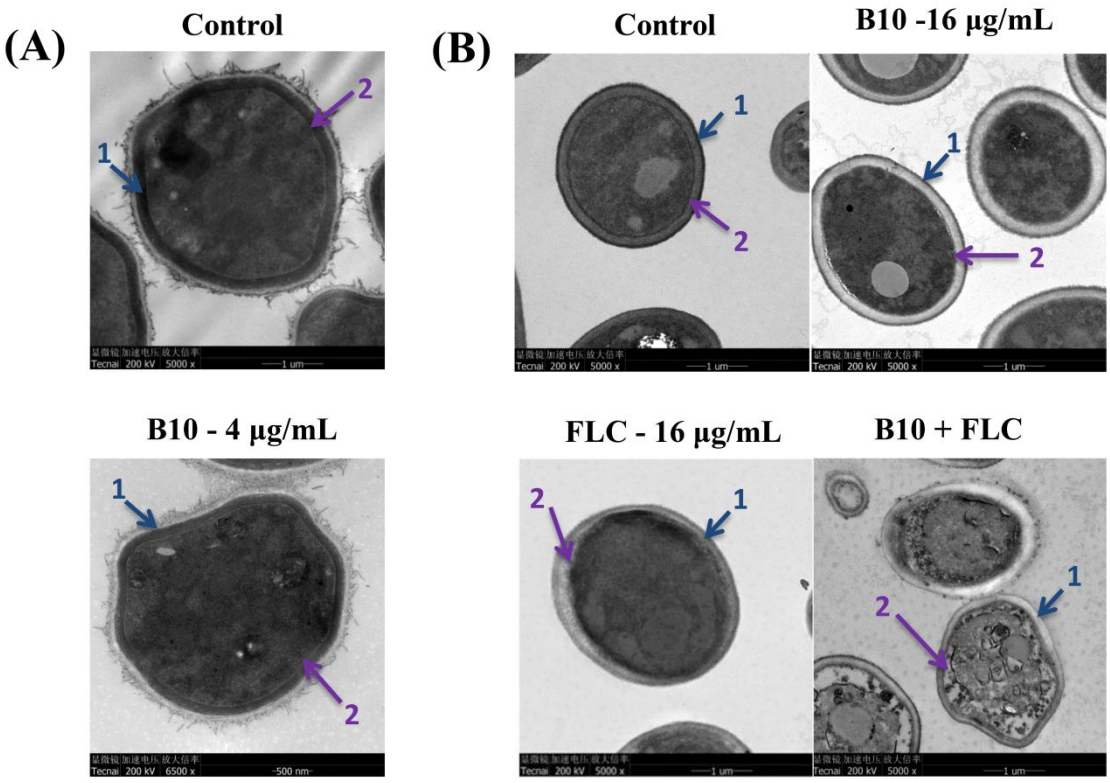


Figure 8. Transmission electron micrographs. The arrows indicated some stuctures as follows: 1, cell wall, 2. cell membrane. (**A**) *C. neoformans* H99 cells treated with or without compound **B10**. (**B**) Drug-resistant *C. albicans* cells (strain number: 0304103) treated with DMSO, FLC or/and compound **B10**.

Evaluation of Efflux of Rhodamine 6G, Sterol Composition Analysis and Real-time RT-PCR Analysis. In order to further explore the mechanism that how compound **B10** synergistically inhibited the drug-resistant fungi, real-time RT-PCR analysis was performed. Lanosterol 14a-demethylase (CYP51), which is encoded by *ERG11* gene, is the target of azoles in the ergosterol biosynthetic pathway.[52] The mutation and high expression of *ERG11* gene in the ergosterol synthesis pathway was considered to be one of the most important mechanisms of *Candida*'s resistance to

azoles.[53] When cells were treated with 16 μg/mL of FLC, the expression of ERG11 was up-regulated about 4-fold stimulated by the antifungal agent (**Figure 9A**). However, when compound **B10** was used in combination with FLC, the up-regulation effect was reversed (**Figure 9A**). The results suggested that compound **B10** could reverse the overexpression of *ERG11* ($P < 0.01$) when it was used in combination with FLC, and reduced the resistance of *C. albicans* to azoles.

The overexpression of efflux pumps is another important mechanism causing drug resistance of *Candida* spp., and *MDR1* is one of the efflux pumps that involved in drug resistance of *C. albicans*.[54] As shown in **Figure 9A**, the expression of *MDR1* was up-regulated by 2-fold when treated with 16 μg/mL of FLC. And when treated with compound **B10** and FLC in combination, the expression of *MDR1* was significantly down-regulated ($P < 0.001$， **Figure 9A**). Therefore, compound **B10** could synergistically inhibit the efflux pumps and increase the drug concentration in the cells, leading to the decline of resistance to azole antifungal agents.

In order to explore the effect of compound **B10** on the efflux pump of drug-resistant *C. albicans*, the fungal efflux efficiency of fluorescent compound rhodamine 6G was evaluated. As shown in **Figure 9B**, when no glucose (GLC) was added, the efflux of rhodamine 6G in the fungal cells was significantly increased after the treatment with the combination of FLC and compound **B10**. The results were consistent with the fact that the damage of cell membrane (**Figure 9B**) was able to cause the exudation of intracellular contents. Also, it was found that when GLC was added, there was no significant difference among the total efflux amounts of

rhodamine 6G in the three groups ($P > 0.05$). The D-value between the groups with or without GLC reflects the efficiency of ATP-depended efflux pump of fungal cells. Comparing the D-value between the FLC and FLC/**B10** group indicated that the active ATP-depended efflux of rhodamine 6G was significantly reduced when treated with FLC and compound **B10** in combination ($P < 0.01$). Surprisingly, the active ATP-depended efflux of rhodamine 6G was also reduced when the drug-resistant *C. albicans* was treated with FLC alone, which might be caused by the damage of cell membrane by high concentration of FLC (**Figure 8B**) because drug efflux pumps are a class of proteins that exist on cell membrane.

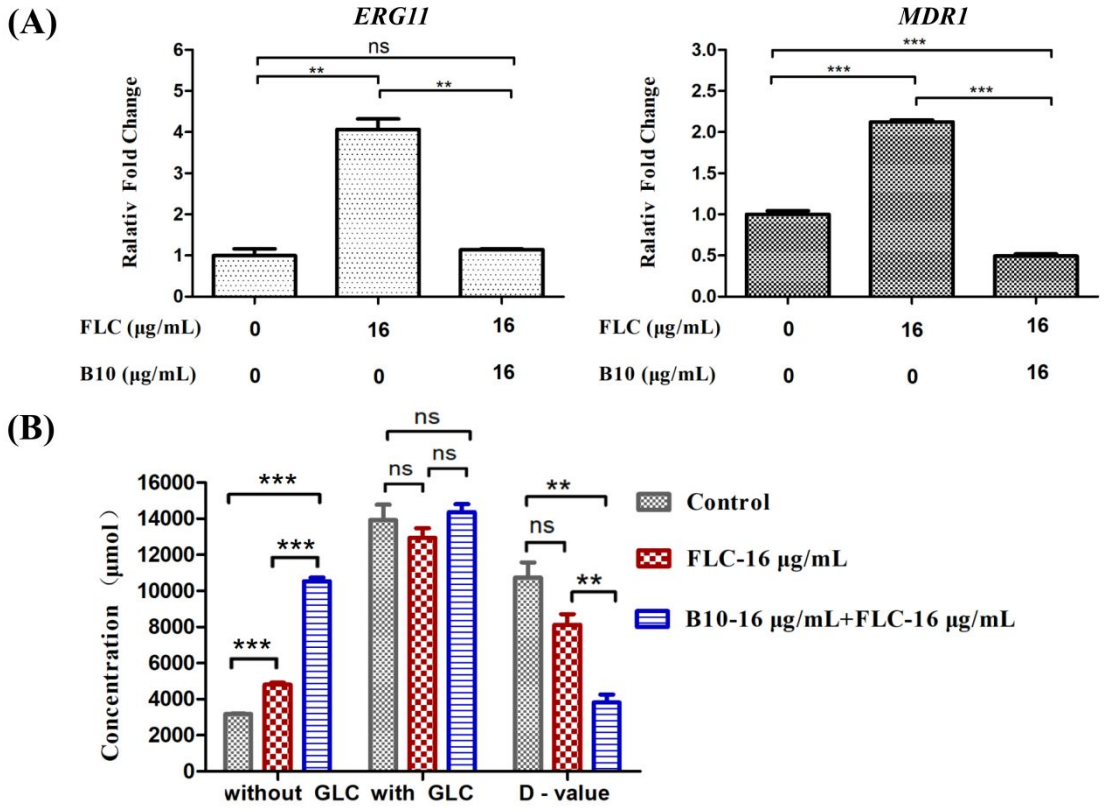


Figure 9. Real-time RT-PCR analysis of *ERG11*, *MDR1* expression (**A**) and the efflux of rhodamine 6G (**B**) of *C. albicans* (strain number: 0304103). Gene expression is indicated as a fold change relative to *ACT1*. All gene expression levels and the

efflux of rhodamine 6G were measured in triplicate, and the data were shown as mean ± standard deviation. Statistical significance among groups was determined by Student's t test (** $P < 0.01$, *** $P < 0.001$).

The sterol composition analysis (**Table 5**) revealed that when treated with 16 μg/mL of FLC, the production of ergosterol was reduced (from 68.79% to 24.14%), and a high amount of obtusifoliol (14.64%) and eburicol (31.67%) were observed. The results were consistent with the TEM analysis that high concentration of FLC could slightly damage the cell membrane (**Figure 8B**). When 16 μg/mL of compound **B10** was used in combination with FLC, the production of ergosterol was further reduced (13.45%), and the production of lanosterol and several unknown sterols were increased. Moreover, when compound **B10** was used alone, no obvious change was observed comparing with the untreated group. These results indicated that compound **B10** could affect the ergosterol biosynthetic pathway of drug-resistant *C. albicans* when used in combination with FLC.

Table 5. Sterol composition of untreated and FLC or/and compound B10 treated *C. albicans* (strain number: 0304103). [a]

Sterol	Retention time (min)	Groups			
		Control	B10	FLC	B10 + FLC
Ergosterol (1)	14.02	68.79%	74.34%	24.14%	13.45%
Obtusifoliol (2)	15.13	ND	ND	14.64%	15.63%
Lanosterol (3)	15.67	18.89%	13.69%	15.44%	22.11%
Eburicol (4)	16.49	ND	ND	31.67%	27.88%
Unknown sterol (5)	13.49	5.30%	3.41%	1.59%	6.48%
Unknown sterol (6)	13.59	ND	ND	6.83%	6.62%
Unknown sterol (7)	14.48	7.02%	8.55%	2.70%	7.82%

[a] ND = Not Determined

On the basis of the above results, when compound **B10** was used in combination with FLC, it could reverse the overexpression of *ERG11* and down-regulate the expression of *MDR1*, thereby affecting the ergosterol biosynthetic pathway and the efficiency of efflux pump. As a result, the underlying mechanism of the synergistic effect of compound **B10** against drug-resistant fungi is associated with reducing the level of CYP51 and increasing the concentration of FLC in the cells. Transmission electron microscopy analysis also confirmed that compound **B10** restored the damaging effect of FLC on cell membrane (**Figure 8B**).

Inhibitory Activity of Compound B10 towards Dopamine D2 Receptor. Haloperidol is a typical butyrophenone type antipsychotic, and it has a high possibility to cause severe extrapyramidal symptoms in clinic as a result of its strong inhibitory effect on dopamine D2 receptor (D2R).[55] Whether compound **B10** binds to the same molecular target as haloperidol was further investigated by detecting the production of cyclic AMP (cAMP), using the homogeneous time-resolved fluorescence (HTRF) assay.[56] The results indicated that the D2R inhibitory activity of compound **B10** was dramatically reduced in comparison with that of haloperidol (**Table 6**). Thus, compound **B10** may have low risks to cause haloperidol-related side effects such as extrapyramidal symptoms. Moreover, the main molecular target of compound **B10** still remains to be further confirmed.

Table 6. The inhibitory activity of Risperidone, Haloperidol, compound **A1** and **B10** on dopamine D2 receptor [a]

Compounds	RIS [a]	HAL [a]	A1	B10
IC_{50} (nM)	1.5	0.8	217.0	679.6

[a] Abbreviations: RIS, Risperidone, HAL, Haloperidol.

CONCLUSIONS

Starting from haloperidol, an antifungal lead compound identified from drug repurposing, a series of novel benzocyclane derivatives were designed, synthesized and assayed, which showed improved antifungal potency and antifungal spectrum. In particular, compound **B10** was identified to be a promising lead compound for the development of new generation of antifungal agents. It showed good inhibitory activities against a variety of fungal pathogens including *C. albicans*, *C. glabrata* and *C. neoformans*. Compound **B10** was also able to synergize with FLC to treat resistant *C. albicans* infections. Its antifungal potency was further validated in the *in vivo* cryptococcosis and candidiasis models. Unlike haloperidol, the D2R inhibitory activity of compound **B10** was substantially decreased, indicating a potential new antifungal mechanism or binding target. Further structural optimization and target verification studies are in progress.

EXPERIMENTAL SECTION

Chemistry. *General methods.* All the reagents and solvents were analytically pure and were used as received from the vendors. TLC analysis was carried out on silica gel plates GF254 (Qindao Haiyang Chemical, China). Silica gel chromatography was

carried out on 300-400 mesh gel. The anhydrous solvents and reagents were dried by routine protocols. NMR spectra were recorded on a Bruker Avance 600 spectrometer (Bruker Company, Germany) using TMS as an internal standard and $CDCl_3$ or DMSO-*d6* as the solvents. The chemical shifts (δ values) and coupling constants (*J* values) are given in ppm and Hz, respectively. The mass spectra were recorded on an API-3000 LC-MS mass spectrometer. The purities of the compounds were determined by HPLC (Agilent 1260), and all final compounds exhibited purities greater than 96%.

4-(4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl)-1-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)butan-1-one (A1). To a stirred solution of compound **4** (215 mg, 0.73 mmol, 1.0 equiv) and compound **5** (231 mg, 1.09 mmol, 1.5 equiv) in DMF (30 mL) was added DIPEA (279 mg, 2.16 mmol, 3.0 equiv). The mixture was stirred at 35 ^oC for 16 h. Then the mixture was diluted with H_2O (30 mL) and extracted with EtOAc (20 mL × 2). The combined organic layers were washed with H_2O (30 mL × 3) and saturated NaCl solution (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (CH_2Cl_2/MeOH/Et_3N = 100/1.5/0.5, v/v/v) to afford target compound **A1** as a white solid (142 mg, 45.1% yield). ^1H NMR (600 MHz, DMSO-d_6) δ: 7.73 (s, 1H), 7.70 (dd, *J* = 7.8, 1.8 Hz, 1H), 7.36 (d, *J* = 8.4 Hz, 2H), 7.33 (d, *J* = 8.4 Hz, 2H), 7.26 (d, *J* = 7.8 Hz, 1H), 4.83 (s, 1H), 2.94 (s, 2H), 2.87-2.80 (m, 4H), 2.63-2.53 (m, 2H), 2.42-2.20 (m, 4H), 1.87-1.75 (m, 4H), 1.68 (s, 2H), 1.56 (s, 4H), 1.51-1.41 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 199.25, 149.03, 148.36,

143.22, 135.13, 130.63, 129.02, 128.20, 127.56, 126.65, 125.79, 69.33, 57.15, 48.74, 37.55, 35.56, 35.46, 31.81, 27.74, 27.57, 21.93. HRMS (ESI, positive) m/z calcd for $C_{26}H_{33}ClNO_2$ $[M + H]^+$ 426.2194, found 426.2211. HPLC purity 97.9%. Retention time: 9.05 min, eluted with 70%-95% methol and 0.1% TFA.

1-(4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl)-4-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)butane-1,4-dione (A2). To a stirred solution of compound **8** (69 mg, 0.28 mmol, 1.0 equiv) and compound **5** (72 mg, 0.34 mmol, 1.2 equiv) in 15 mL of DMF were added Et_3N (141 mg, 1.4 mmol, 5.0 equiv) and HATU (160 mg, 0.42 mmol, 1.5 equiv). The mixture was stirred at room temperature for 3 h. Then the mixture was diluted with H_2O (30 mL) and extracted with EtOAc (20 mL × 2). The combined organic layers were washed with H_2O (30 mL × 3) and saturated NaCl solution (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (CH_2Cl_2/MeOH/Et_3N = 100/1/0.5, v/v/v) to afford **A2** as a white solid (95 mg, 80.1% yield). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 7.73 (s, 1H), 7.71 (dd, J = 7.2, 1.8 Hz, 1H), 7.52-7.50 (m, 2H), 7.40-7.37 (m, 2H), 7.26 (d, J = 7.8 Hz, 1H), 5.24 (s, 1H), 4.28 (d, J = 13.2 Hz, 1H), 3.86 (d, J = 13.2 Hz, 1H), 3.44 (td, J = 13.2, 2.4 Hz, 1H), 3.27-3.13 (m, 2H), 2.94 (td, J = 12.6, 2.4 Hz, 1H), 2.87-2.81 (m, 4H), 2.75-2.70 (m, 2H), 1.95 (td, J = 13.2, 4.2 Hz, 1H), 1.85-1.77 (m, 2H), 1.72 (td, J = 13.2, 4.8 Hz, 1H), 1.64 (dd, J = 13.2, 1.8 Hz, 1H), 1.62-1.52 (m, 5H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 199.13, 169.96, 149.15, 148.90, 143.82, 135.36, 131.51, 129.62, 128.66, 128.31, 127.30, 126.35, 70.44, 41.71, 38.49, 38.10, 37.94, 36.18, 36.14, 33.55,

32.41, 28.32, 28.15, 27.16. HRMS (ESI, positive) m/z calcd for $C_{26}H_{31}ClNO_3$ $[M + H]^+$ 440.1987, found 440.1997.

1-(4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl)-4-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)butan-1-one (A3). To a stirred solution of compound **9** (61 mg, 0.26 mmol, 1.0 equiv) and compound **5** (66 mg, 0.34 mmol, 1.2 equiv) in 15 mL of DMF were added Et_3N (131 mg, 1.3 mmol, 5.0 equiv) and HATU (148 mg, 0.39 mmol, 1.5 equiv). The reaction mixture was stirred at room temperature for 3 h. Then the mixture was diluted with H_2O (30 mL) and extracted with EtOAc (20 mL × 2). The combined organic layers were washed with H_2O (30 mL × 3) and saturated NaCl solution (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (CH_2Cl_2/MeOH/ Et_3N = 100/1/0.5, v/v/v) to afford **A3** as a white solid (98 mg, 79.8% yield).^{1}H NMR (600 MHz, DMSO-d_6) δ: 7.49 (d, J = 7.8 Hz, 2H), 7.37 (d, J = 7.8 Hz, 2H), 6.99 (d, J = 7.8 Hz, 1H), 6.92 (s, 1H), 6.88 (d, J = 7.2 Hz, 1H), 5.22 (s, 1H), 4.34 (d, J = 11.4 Hz, 1H), 3.69 (d, J = 13.2 Hz, 1H), 3.36 (t, J = 12.6 Hz, 1H), 2.94-2.87 (m, 1H), 2.73-2.67 (m, 4H), 2.52 (t, J = 7.2 Hz, 2H), 2.34 (t, J = 7.2 Hz, 2H), 1.85-1.69 (m, 6H), 1.61-1.48 (m, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 170.07, 148.35, 142.72, 140.20, 139.26, 130.99, 128.92, 128.76, 127.77, 126.78, 125.58, 69.88, 41.25, 38.16, 37.41, 37.28, 35.90, 35.43, 34.24, 32.11, 31.75, 28.12), 28.07, 26.72. HRMS (ESI, positive) m/z calcd for $C_{26}H_{33}ClNO_2$ $[M + H]^+$ 426.2194, found 426.2196.

4-(4-Chlorophenyl)-1-(3-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)propyl) piperidin-4-ol hydrochloride (B1). To a solution of compound **2a** (106 mg, 0.4 mmol, 1.0 equiv) and compound **5** (127 mg, 0.6 mmol, 1.5 equiv) in DMF (30 mL) was added DIPEA (155 mg, 1.2 mmol, 3.0 equiv). The reaction mixture was stirred at 35 °C for 16 h. Then the mixture was diluted with H_2O (30 mL) and extracted with EtOAc (20 mL × 2). The combined organic layers were washed washed with H_2O (30 mL × 3), saturated NH_4Cl solution (30 mL) and saturated NaCl solution (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (CH_2Cl_2/MeOH/Et_3N = 100/1.5/0.5, v/v/v) to afford compound **B1** as a white solid (93 mg, 53.4% yield). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.83 (br, 1H), 7.49 (d, J = 9.0 Hz, 2H), 7.43 (d, J = 9.0 Hz, 2H), 7.03 (d, J = 7.8 Hz, 1H), 6.98 (s, 1H), 6.94 (d, J = 7.2 Hz, 1H), 5.57 (s, 1H), 3.47-3.40 (m, 2H), 3.24-3.15 (m, 2H), 3.12-3.06 (m, 2H), 2.75-2.69 (m, 4H), 2.57 (t, J = 7.8 Hz, 2H), 2.45 (td, J = 13.8 Hz, 4.2 Hz, 2H), 2.09-2.01 (m, 2H),1.82-1.73 (m, 4H), 1.60-1.50 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6,) δ: 146.97, 142.92, 140.6, 137.93, 131.43, 128.89, 128.82, 128.02, 126.57, 125.51, 67.91, 55.50, 48.20, 35.88, 35.41, 34.81, 32.09, 31.67, 28.08, 28.02, 24.97. HRMS (ESI, positive) m/z calcd for $C_{25}H_{33}ClNO$ $[M + H]^+$ 398.2245, found 398.2246.

The synthesis of compounds **B2-B14** was similar to compound **B1.**

4-(4-Chlorophenyl)-1-(4-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)butyl)pi peridin-4-ol hydrochloride (B2). White solid (73 mg), yield: 42.6%. ^{1}H NMR (600

MHz, DMSO-d_6,) δ: 10.53 (br, 1H), 7.47 (d, J = 9.0 Hz, 2H), 7.43 (d, J = 9.0 Hz, 2H), 7.00 (d, J = 7.2 Hz, 1H), 6.95 (s, 1H), 6.90 (d, J = 7.2 Hz, 1H), 5.56 (s, 1H), 3.42-3.35 (m, 2H), 3.22-3.14 (m, 2H), 3.14-3.06 (m, 2H), 2.74-2.68 (m, 4H), 2.57-2.51 (m, 2H), 2.44-2.33 (s, 2H), 1.82-1.67 (m, 6H), 1.63-1.50 (m, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 147.01, 142.78, 140.30, 138.97, 131.42, 128.90, 128.80, 128.00, 126.58, 125.56, 67.95, 55.51, 48.11, 35.88, 35.42, 34.77, 34.11, 32.10, 28.11, 28.04, 22.94. HRMS (ESI, positive) m/z calcd for $C_{26}H_{35}ClNO$ $[M + H]^+$ 412.2402, found 412.2407.

4-(4-Chlorophenyl)-1-(5-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)pentyl)piperidin-4-ol hydrochloride (B3). White solid (51 mg), yield: 42.2%. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.87 (br, 1H), 7.48 (d, J = 8.4 Hz, 2H), 7.41 (d, J = 8.4 Hz, 2H), 6.98 (d, J = 7.2 Hz, 1H), 6.92 (s, 1H), 6.84 (d, J = 7.2 Hz, 1H), 5.56 (s, 1H), 3.40-3.34 (m, 2H), 3.22-3.13 (m, 2H), 3.07-3.00 (m, 2H), 2.74-2.65 (m, 4H), 2.53-2.49 (m, 2H), 2.48-2.41 (m, 2H), 1.82-1.71 (m, 6H), 1.61-1.47 (m, 6H) , 1.36-1.27 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 147.03, 142.73, 140.17, 139.39, 131.44, 128.93, 128.77, 128.03, 126.62, 125.59, 68.01, 55.58, 48.09, 35.92, 35.46, 34.80, 34.44, 32.14, 30.45, 28.15, 28.10, 25.83, 23.00. HRMS (ESI, positive) m/z calcd for $C_{27}H_{37}ClNO$ $[M + H]^+$ 426.2558, found 426.2569.

4-(4-Chlorophenyl)-1-(6-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)hexyl)piperidin-4-ol hydrochloride (B4). White solid (70 mg), yield: 43.0%. ^{1}H NMR (600 MHz, DMSO-d_6,) δ: 10.84 (br, 1H), 7.49 (d, J = 9.0 Hz, 2H), 7.43 (d, J = 9.0 Hz, 2H), 6.98 (d, J = 7.8 Hz, 1H), 6.92 (s, 1H), 6.87 (dd, J = 7.2 Hz, 1.2 Hz, 1H), 5.55 (s, 1H),

3.41-3.36 (m, 2H), 3.22-3.14 (m, 2H), 3.07-3.00 (m, 2H), 2.73-2.68 (m, 4H), 2.51-2.48 (m, 2H), 2.48-2.42 (m, 2H), 1.81-1.70 (m, 6H), 1.60-1.49 (m, 6H) , 1.36-1.30 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 147.03, 142.71, 140.11, 139.57, 131.45, 128.90, 128.75, 128.03, 126.61, 125.54, 67.99, 55.71, 48.11, 35.91, 35.45, 34.80, 34.62, 32.14, 30.73, 28.24, 28.16, 27.90, 28.10, 26.01, 23.13. HRMS (ESI, positive) m/z calcd for $C_{28}H_{39}ClNO$ $[M + H]^+$ 440.2715, found 440.2721.

4-(4-Chlorophenyl)-1-(7-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)heptyl)piperidin-4-ol hydrochloride (B5). White solid (38 mg), yield: 38.6%. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.65 (s, 1H), 7.49 (d, J = 6.6 Hz, 2H), 7.43 (d, J = 7.8 Hz, 2H), 6.98 (d, J = 7.2 Hz, 1H), 6.91 (s, 1H), 6.86 (d, J = 7.2 Hz, 1H), 5.54 (s, 1H), 3.36-3.50 (m, 2H), 3.20-3.09 (m, 2H), 3.09-2.90 (m, 2H), 2.75-2.66 (m, 4H), 2.52-2.46 (m, 2H), 2.45-2.31 (m, 2H), 1.82-1.66 (m, 6H), 1.60-1.49 (m, 6H), 1.37-1.25 (m, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 147.11, 142.69, 140.07, 139.65, 131.42, 128.87, 128.74, 128.01, 126.64, 125.51, 68.07, 55.77, 48.13, 35.91, 35.45, 34.92, 34.69, 32.15, 30.92, 28.53, 28.37, 28.15, 28.10, 26.14, 23.35. HRMS (ESI, positive) m/z calcd for $C_{29}H_{41}ClNO$ $[M + H]^+$ 454.2871, found 454.2883.

1-(4-Chlorophenyl)-4-(4-(2,3-dihydro-1*H*-inden-5-yl)butyl)cyclohexan-1-ol hydrochloride (B6). White solid (47 mg), yield: 39.6%. ^{1}H NMR (600 MHz, DMSO-*d6*) δ: 10.37 (s, 1H), 7.48 (d, J = 8.4 Hz, 2H), 7.43 (d, J = 8.4 Hz, 2H), 7.13 (d, J = 7.8 Hz, 1H), 7.08 (s, 1H), 6.96 (d, J = 7.8 Hz, 1H), 5.56 (s, 1H), 3.38 (d, J = 11.4 Hz, 2H), 3.18 (d, J = 11.4 Hz, 2H), 3.14-3.06 (m, 2H), 2.85-2.78 (m, 4H), 2.58 (t, J = 7.8 Hz, 2H), 2.37 (t, J = 12.0 Hz, 2H), 1.99 (p, J = 7.2 Hz, 2H), 1.78 (d, J = 13.2 Hz,

2H), 1.76-1.68 (m, 2H), 1.63-1.56 (m, 2H). ^{13}C NMR (150 MHz, DMSO-*d6*) δ: 147.47, 144.25, 141.53, 139.80, 131.96, 128.54, 127.09, 126.62, 124.69, 124.47, 68.42, 56.08, 48.67, 35.33, 34.91, 32.73, 32.39, 28.83, 25.58, 23.40. HRMS (ESI, positive) m/z calcd for $C_{24}H_{31}ClNO$ $[M + H]^+$ 384.2089, found 384.2094.

1-(4-Chlorophenyl)-4-(4-(5,6,7,8-tetrahydronaphthalen-2-yl)butyl)cyclohexan-1-ol hydrochloride (B7). White solid (55 mg), yield: 37.1%. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.63 (s, 1H), 7.50 (s, 2H), 7.45 (s, 2H), 7.01-6.85 (m, 3H), 5.56 (s, 1H), 3.22-3.11 (m, 2H), 3.12-2.99 (m, 2H), 2.74-2.62 (m, 4H), 2.58-2.52 (m, 2H), 2.46-2.31 (m, 2H), 1.90-1.48 (m, 12H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 146.90, 138.42, 136.30, 133.91, 131.45, 128.77, 128.68, 128.02, 126.57, 125.46, 67.87, 55.62, 48.20, 34.85, 34.12, 28.75, 28.36, 28.07, 22.82, 22.76. HRMS (ESI, positive) m/z calcd for $C_{25}H_{33}ClNO$ $[M + H]^+$ 398.2245, found 398.2252.

1-(4-Chlorophenyl)-4-(4-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)butyl)piperazine hydrochloride (B8). White solid (60 mg), yield: 50.1%. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.71 (s, 1H), 7.40 (d, J = 8.4 Hz, 2H), 7.27 (d, J = 8.5 Hz, 2H), 7.00 (d, J = 7.8 Hz, 1H), 6.95 (d, J = 1.8 Hz, 1H), 6.91 (dd, J = 7.8, 1.8 Hz, 1H), 3.51 (d, J = 11.4 Hz, 2H), 3.08-3.02 (m, 2H), 3.02-2.94 (m, 2H), 2.87-2.79 (m, 1H), 2.74-2.68 (m, 4H), 2.56-2.51 (m, 2H), 2.14-2.04 (m, 2H), 1.94 (s, 1H), 1.92 (s, 1H), 1.82-1.71 (m, 4H), 1.62-1.50 (m, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 143.78, 143.30, 140.83, 139.47, 131.57, 129.41, 129.32, 128.98, 128.97, 126.08, 56.21, 52.20, 38.65, 36.40, 35.94, 34.64, 32.62, 30.02, 28.64, 28.62, 28.56, 23.37. HRMS (ESI, positive) m/z calcd for $C_{25}H_{34}ClN_2$ $[M + H]^+$ 397.2405, found 397.2414.

4-(4-Chlorophenyl)-1-(4-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)butyl)piperidine hydrochloride (B9). White solid (43 mg), yield: 35.7%. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.06 (br, 1H), 7.40 (d, J = 8.4 Hz, 2H), 7.26 (d, J = 8.4 Hz, 2H), 7.01 (d, J = 7.8 Hz, 1H), 6.95 (s, 1H), 6.91 (d, J = 7.2 Hz, 1H), 3.53 (d, J = 11.6 Hz, 2H), 3.10-3.04 (m, 2H), 3.03-2.94 (m, 2H), 2.87-2.79 (m, 1H), 2.75-2.69 (m, 4H), 2.54 (t, J = 7.8 Hz, 2H), 2.03-1.91 (m, 4H), 1.81-1.75 (m, 2H), 1.75-1.68 (m, 2H), 1.63-1.50 (m, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 143.78, 143.03, 140.83, 139.47, 131.57, 129.41, 129.32, 128.98, 128.97, 126.07, 56.20, 52.19, 38.66, 36.40, 35.94, 34.64, 32.62, 30.02, 28.64, 28.62, 28.57, 23.36. HRMS (ESI, positive) m/z calcd for $C_{26}H_{36}ClN$ $[M + H]^+$ 396.2453, found 396.2459.

4-(4-Fluorophenyl)-1-(4-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)butyl)piperidin-4-ol hydrochloride (B10). White solid (62 mg), yield: 49.8%. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.34 (s, 1H), 7.54-7.46 (m, 2H), 7.19 (t, J = 8.4 Hz, 2H), 7.01 (d, J = 7.2 Hz, 1H), 6.96 (s, 1H), 6.91 (d, J = 7.8 Hz, 1H), 5.52 (s, 1H), 3.50-3.33 (m, 2H), 3.16 (s, 2H), 3.08 (s, 2H), 2.72 (dd, J = 10.8, 6.6 Hz, 4H), 2.55 (t, J = 7.2 Hz, 2H), 2.40-2.30 (m, 2H), 1.81-1.74 (m, 4H), 1.76-1.69 (m, 2H), 1.63-1.57 (m, 2H), 1.58-1.50 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 161.60, (d, J = 241.5 Hz), 144.66, 143.31, 140.84, 139.50, 129.42, 129.32, 127.17, 126.09, 115.23 (d, J = 21.0 Hz), 68.37, 56.20, 48.76, 36.39, 35.93, 35.59, 34.61, 32.61, 28.62, 28.57, 23.66. HRMS (ESI, positive) m/z calcd for $C_{26}H_{35}FNO$ $[M + H]^+$ 396.2697, found 396.2707. HPLC purity 96.9%. Retention time: 8.88 min, eluted with 70%-95% methol and 0.1% TFA.

1-(4-(6,7,8,9-Tetrahydro-5*H*-benzo[7]annulen-2-yl)butyl)-4-(4-(trifluoromethyl) phenyl)piperidin-4-ol hydrochloride (B11). White solid (78 mg), yield: 50.9%. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.23 (br, 1H), 7.75 (d, J = 7.2 Hz, 2H), 7.69 (d, J = 7.8 Hz, 2H), 7.01 (d, J = 7.8 Hz, 1H), 6.96 (s, 1H), 6.91 (d, J = 7.2 Hz, 1H), 5.70 (s, 1H), 3.41 (s, 2H), 3.22 (s, 2H), 3.13 (s, 2H), 2.76-2.67 (m, 4H), 2.55 (t, J = 7.2 Hz, 2H), 2.45-2.34 (m, 2H), 1.86-1.68 (m, 6H), 1.65-1.49 (m, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 152.76, 142.76, 140.28, 139.01, 128.89, 128.78, 127.50 (q, J = 23.6 Hz), 125.55, 125.18, 125.00, 123.38, 68.38, 55.71, 48.12, 35.87, 35.41, 34.85, 34.13, 32.08, 28.10, 28.04, 23.16. HRMS (ESI, positive) m/z calcd for $C_{27}H_{35}F_3NO$ $[M + H]^+$ 446.2665, found 446.2672.

4-(Pyridin-3-yl)-1-(4-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)butyl)piperi din-4-ol hydrochloride (B12). White solid (38 mg), yield: 30.7%. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.49 (s, 1H), 8.71 (s, 1H), 8.49 (d, J = 4.2 Hz, 1H), 7.85 (d, J = 7.8 Hz, 1H), 7.41 (dd, J = 8.0, 4.8 Hz, 1H), 7.01 (d, J = 7.8 Hz, 1H), 6.96 (s, 1H), 6.91 (dd, J = 7.2, 1.2 Hz, 1H), 5.73 (s, 1H), 3.43 (s, 2H), 3.21 (s, 2H), 3.12 (s, 2H), 2.75-2.69 (m, 4H), 2.55 (t, J = 7.8 Hz, 2H), 2.44 (t, J = 12.0 Hz, 2H), 1.85 (d, J = 13.8 Hz, 2H), 1.81-1.71 (m, 4H), 1.64-1.58 (m, 2H), 1.55 (s, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 148.15, 146.46, 142.87, 140.39, 139.05, 132.60, 128.97, 128.88, 125.64, 123.26, 67.25, 55.70, 48.08, 45.51, 35.95, 35.48, 34.80, 34.16, 32.16, 28.17, 28.12, 23.11. HRMS (ESI, positive) m/z calcd for $C_{25}H_{35}N_2O$ $[M + H]^+$ 379.2744, found 379.2755. HPLC purity 97.7%. Retention time: 7.86 min, eluted with 70%-95% methol and 0.1% TFA.

1-(4-(6,7,8,9-Tetrahydro-5*H*-benzo[7]annulen-2-yl)butyl)-4-(p-tolyl)piperidin-4-ol hydrochloride (B13).White solid (99 mg), yield: 48.0%. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.12 (br, 1H), 7.34 (d, J = 7.8 Hz, 2H), 7.17 (d, J = 7.8 Hz, 2H), 7.01 (d, J = 7.8 Hz, 1H), 6.96 (s, 1H), 6.91 (d, J = 7.2 Hz, 1H), 5.38 (s, 1H), 3.41-3.34 (m, 2H), 3.24-3.14 (m, 2H), 3.11 (s, 2H), 2.75-2.68 (m, 4H), 2.55 (t, J = 7.8 Hz, 2H), 2.35-2.28 (m, 2H), 2.28 (s, 3H), 1.83-1.67 (m, 6H), 1.62-1.56 (m, 2H), 1.58-1.49 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 145.08, 142.82, 140.34, 139.02, 135.81, 128.94, 128.84, 128.60, 125.60, 124.47, 67.92, 55.62, 48.27, 35.91, 35.45, 35.05, 34.15, 32.14, 28.14, 28.08, 23.05, 20.55. HRMS (ESI, positive) m/z calcd for $C_{27}H_{38}NO$ $[M + H]^+$ 392.2948, found 392.2956.

4-([1,1'-Biphenyl]-4-yl)-1-(4-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)butyl)piperidin-4-ol hydrochloride (B14). White solid (130 mg), yield: 48.9%. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 9.85 (s, 1H), 7.66 (d, J = 7.2 Hz, 4H), 7.55 (d, J = 7.8 Hz, 2H), 7.46 (t, J = 7.2 Hz, 2H), 7.36 (t, J = 7.2 Hz, 1H), 7.01 (d, J = 7.2 Hz, 1H), 6.96 (s, 1H), 6.91 (d, J = 7.2 Hz, 1H), 5.50 (s, 1H), 3.50-3.34 (m, 2H), 3.27-3.01 (m, 4H), 2.75-2.69 (m, 4H), 2.55 (t, J = 7.2 Hz, 2H), 2.41-2.18 (m, 2H), 1.89-1.65 (m, 6H), 1.63-1.51 (m, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 142.82, 140.32, 139.91, 139.17, 138.62, 128.94, 127.35, 126.59, 126.39, 125.62, 125.30, 68.34, 56.05, 48.46, 35.92, 35.46, 34.24, 32.14, 28.14, 23.81. HRMS (ESI, positive) m/z calcd for $C_{32}H_{40}NO$ $[M + H]^+$ 454.3104, found 454.3113.

4-(4-Methoxyphenyl)-1-(4-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)butyl) piperidin-4-ol hydrochloride (B15). To a stirred solution of 1-benzyl-4-(4-

methoxyphenyl)piperidin-4-ol (compound **15a**, 110 mg, 0.37 mmol, 1.0 equiv) in 20 mL of MeOH was added $Pd(OH)_2$-C (10.5 mg, catalytic). After being degassed with H_2 ballon, the reaction mixture was stirred at 35 °C under H_2 overnight. Then the reaction mixture was filtered and the filter cake was washed with MeOH (20 mL × 2), the filtrate was concentrated under reduced pressure.The crude product was dissolved with DMF (30 mL), the obtained mixture was added compound **3b** (98 mg, 0.37 mmol, 1.0 equiv) and DIPEA (143 mg, 1.11 mmol, 3.0 equiv), stirred at 60 °C for 16 h. Then the mixture was diluted with H_2O (30 mL) and extracted with EtOAc (20 mL × 2). The combined organic layers were washed with H_2O (30 mL × 3), saturated NH_4Cl solution (30 mL) and saturated NaCl solution (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (CH_2Cl_2/MeOH/Et_3N = 100/1.5/0.5, v/v/v) to afford compound **B15** as a white solid (82 mg, 49.8% yield). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.68 (s, 1H), 7.38 (d, J = 9.0 Hz, 2H), 7.00 (d, J = 7.2 Hz, 1H), 6.95 (d, J = 1.2 Hz, 1H), 6.93-6.89 (m, 3H), 5.36 (s, 1H), 3.74 (s, 3H), 3.38-3.33 (m, 2H), 3.21-3.12 (m, 2H), 3.11-3.03 (m, 2H), 2.71 (dd, J = 10.8, 6.6 Hz, 4H), 2.54 (d, J = 7.8 Hz, 2H), 2.45-2.34 (m, 2H), 1.81-1.70 (m, 6H), 1.63-1.50 (m, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 158.08, 142.79, 140.31, 140.03, 138.99, 128.91, 128.81, 125.70, 125.57, 113.36, 67.66, 55.57, 55.04, 48.26, 35.89, 35.43, 35.07, 34.12, 32.11, 28.11, 28.06, 22.99. HRMS (ESI, positive) m/z calcd for $C_{27}H_{38}NO_2$ $[M + H]^+$ 408.2897, found 408.2906.

4-(Naphthalen-2-yl)-1-(4-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)butyl)piperidin-4-ol hydrochloride (B16). White solid (118 mg), yield: 45.2%. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.86 (br, 1H), 7.99 (s, 1H), 7.94-7.88 (m, 3H), 7.64 (d, J = 7.8 Hz, 1H), 7.54-7.47 (m, 2H), 7.00 (d, J = 7.8 Hz, 1H), 6.96 (d, J = 1.2 Hz, 1H), 6.91 (dd, J = 7.8, 1.2 Hz, 1H), 5.62 (s, 1H), 3.39 (s, 2H), 3.24 (s, 2H), 3.10 (s, 2H), 2.71 (dd, J = 10.8, 7.8 Hz, 4H), 2.55 (t, J = 7.8 Hz, 4H), 1.87 (d, J = 13.8 Hz, 2H), 1.82-1.73 (m, 4H), 1.64-1.57 (m, 2H), 1.58-1.49 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 145.47, 142.78, 140.30, 139.01, 132.61, 131.97, 128.91, 128.80, 127.96, 127.65, 127.32, 126.12, 125.80, 125.56, 123.55, 122.76, 68.29, 55.64, 48.22, 35.89, 35.43, 34.81, 34.14, 32.10, 28.15, 28.11, 28.05, 23.07. HRMS (ESI, positive) m/z calcd for $C_{30}H_{38}NO$ $[M + H]^+$ 428.2948, found 428.2955.

4-(Naphthalen-1-yl)-1-(4-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)butyl)piperidin-4-ol hydrochloride (B17). White solid (86 mg), yield: 40.9%. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.35 (s, 1H), 8.93-8.87 (m, 1H), 7.96-7.90 (m, 1H), 7.85 (d, J = 8.4 Hz, 1H), 7.56 (d, J = 7.2 Hz, 1H), 7.53-7.44 (m, 3H), 7.00 (d, J = 7.2 Hz, 1H), 6.95 (s, 1H), 6.90 (d, J = 7.2 Hz, 1H), 5.76 (s, 1H), 3.49-3.33 (m, 2H), 3.10 (s, 2H), 2.79-2.64 (m, 4H), 2.54 (t, J = 7.2 Hz, 2H), 2.24 (d, J = 13.2 Hz, 2H), 1.81-1.69 (m, 4H), 1.63-1.57 (m, 2H), 1.57-1.49 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 142.80, 142.24, 140.31, 139.03, 134.42, 130.64, 128.93, 128.82, 128.46, 127.03, 125.59, 125.17, 124.95, 122.71, 69.24, 55.77, 48.05, 35.90, 35.44, 34.35, 34.16, 32.12, 28.17, 28.12, 28.07, 23.17. HRMS (ESI, positive) m/z calcd for $C_{30}H_{38}NO$ $[M + H]^+$ 428.2948, found 428.2947.

4-(4-Morpholinophenyl)-1-(4-(6,7,8,9-tetrahydro-5*H*-benzo[7]annulen-2-yl)butyl)piperidin-4-ol hydrochloride (B18). White solid (84 mg), yield: 44.2%. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 9.68 (s, 1H), 7.30 (d, J = 8.4 Hz, 2H), 7.01 (d, J = 7.2 Hz, 1H), 6.96 (s, 1H), 6.93 (d, J = 8.4 Hz, 2H), 6.91 (d, J = 7.2 Hz, 1H), 5.28 (s, 1H), 3.75-3.72 (m, 4H), 3.38 (d, J = 11.4 Hz, 2H), 3.23-3.15 (m, 2H), 3.14-3.10 (m, 2H), 3.09-3.06 (m, 4H), 2.74-2.70 (m, 4H), 2.55 (t, J = 7.8 Hz, 2H), 2.26-2.17 (m, 2H), 1.82-1.75 (m, 4H), 1.74-1.67 (m, 2H), 1.63-1.51 (m, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ: 149.91, 142.80, 140.33, 138.99, 138.61, 128.92, 128.82, 125.58, 125.18, 114.67, 67.54, 66.07, 55.59, 48.56, 48.29, 35.89, 35.43, 35.04, 34.12, 32.12, 28.12, 28.06, 23.00. HRMS (ESI, positive) m/z calcd for $C_{30}H_{43}N_2O_2$ $[M + H]^+$ 463.3319, found 463.3318.

Strains, culture and agents. Strains were incubated in YPD (1% yeast extract, 2% peptone and 2% dextrose) at 30°C in a shaking incubator. *C. albicans* SC5314, *C. tropicalis* 3890, *C. parapsilosis* 20090, *C. krusei* 4996, *C. glabrata* 8535, *C. neoformans* H99 and drug-resistant *C. albicans* (strains number: 0304103, 4108 and 7781) were provided by Changzheng Hospital of Shanghai, China, *C. neoformans* ATCC 34877 was obtained from American Type Culture Collection (ATCC). All compounds were dissolved in DMSO at 10 mg/mL as stock solutions.

Antifungal Susceptibility Testing. The test was performed using the plate microdilution method according to according to CLSI (M27-A).[38] *C. albicans* cells during the exponential growth phase were harvested and resuspended in RPMI 1640 medium to a concentration of 1×10^3 CFU/mL. Different concentrations of FLC and

compounds were added to the fungal suspension in 96-well plates, and then the plates were incubated for 48 h at 35 °C. The inhibitory effect towards *C. neoformans* was indicated by detecting the optical density at 630 nm with a spectrophotometer. The MIC_{50} value was the lowest concentration of the compound when at least 50% of the growth of cells was inhibited, compared with the growth of the drug-free wells. Each compound was tested in triplicate. And the inhibitory effects towards other fungi strains were tested by the same method, except that *C. neoformans* cells were incubated for 72 h instead of 48 h at 35 °C.

Checkerboard Microdilution Assay. Assays were performed according to the methods of the CLSI (formerly NCCLS) (M27-A).[39] Briefly, drug-resistant *C. albicans* cells during the exponential growth phase were harvested and re-suspended in RPMI 1640 medium to a concentration of 1×10^3 CFU/mL, then the suspension was transferred to 96-well plates. And then FLC and compound **B10** were each serially double-diluted in the 96-well plates, starting at 64 μg/mL and ending at 2 μg/mL or 0.125 μg/mL respectively. A group of drug-free medium with fungi cells and a group of drug-free medium without fungi cells were used as the positive and negative controls, respectively. The plates were incubated for 48 h at 35 °C, and the MIC_{50} were determined as described above. The data obtained were analyzed using the fractional inhibitory concentration index (FICI) method based on the Loewe additivity theory.[57] Synergy was defined as FIC index ≤0.5, antagonism was defined as FIC index > 4, and when 0.5 < FIC index ≤4 was considered indifferent.[39]

Growth Curve Assay. Growth curve assay was performed according to the reported protocol with a few modifications.[41] *C. neoformans* or *C. albicans* cells during the exponential growth phase were harvested and re-suspended in RPMI 1640 medium to a concentration of 1×10^6 CFU/mL, and then different concentrations of FLC or/and compound **B10** were added. The cells were cultured at 30 °C with constant shaking (200 rpm.) and the number of cells were counted at designated time points (0, 4, 8, 12, 24 and 48 h). The control group wasn't treated with FLC or compound.

Melanin and urease production assay. Melanin and urease production assay were performed according to the reported protocol[42] with a few modifications. *C. neoformans* cells during the exponential growth phase were harvested and resuspended to 1×10^6 CFU/mL in RPMI 1640 medium with different concentrations of FLC**,** compound **B10** and compound **B12**. Cells were transferred to *L*-DOPA or urea medium, and incubated for 36 h at 30 ^{o}C to detect melanin or urease production.

The Evaluation of the Production of Capsular Polysaccharides. The evaluation of the production of capsular polysaccharides was performed according to the reported protocol[58] with a few modifications. A colony of *C. neoformans* was inoculated in 100 mL of Yeast Nitrogen Base (YNB) medium, and cultured for 4 days at 30 °C with constant shaking (200 rpm). The *C. neoformans* cells were spinned down (9000 rpm, 10 min) and weighed, as the supernatant was collected. Then 3.0 volumes of EtOH were added slowly to the supernatant as a white precipitate appeared. The suspension was stayed still overnight at 4 °C, then the precipitate was

collected by centrifugation and air dried to remove EtOH. The precipitate was dissolved with 3 mL of double distilled water (ddH2O), and that was the capsular polysaccharides (GXM and GalXM). The polysaccharide concentration was determined by the phenol sulfuric method.[59] ***In vitro*** **Filamentation Assay**. *In vitro* filamentation assay was performed according to the reported protocol.[43] *C. albicans* was harvested as described above, washed with phosphate buffer saline (PBS) for three times, and re-suspended to a concentration of 1×10^6 CFU/mL in RPMI 1640 medium. The suspension was tranfered to 12-well plates while each well was added 2 mL of suspension, and then FLC and different concentrations of Compound **B10** were added. Plates were incubated for 3 h at 37 °C and the difference of morphology between treated and untreated cells were examined by light microscophy. Microscopy was performed using differential interference contrast (DIC) on an Axio observer D1 inverted microscope (Carl Zeiss, Inc., Thornwood, NY) equipped for photography. The filamentation assay in the solid spider medium was performed according to a similar protocol except that the plates were incubated for about 7 days at 37 °C.

In vitro **Biofilm Formation Assay**. According to the well-established protocol[51], the assay was performed in a 96-well tissue culture plate (Corning, cat. number: 3599), which was seeded with 100 μL of cell suspensions (1×10^6 CFU/mL) in RPMI 1640 medium for each experiment well. Then the cells were cultured statically for 90 min at 37 °C, and the medium was removed. Each well was washed with 100 μL of PBS to remove the non-adherent cells, and different concentrations of FLC and compound **B10** in RPMI 1640 medium were added. The cells were incubated for another 24 h at

37°C. A semiquantitative measure of the biofilm formation was calculated using the XTT reduction assay.[44]

***In vivo* Antifungal Potency.** The experimental procedures, the animal use and care protocols were approved by the Committee on Ethical Use of Animals of Second Military Medical University. ICR female mice (4~6 weeks old, weighs 18~20 g) were obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences. The mice were kept for 7 days before inoculation. Drug solutions of FLC (2 mg/kg in NS) and compound **B10** (12 mg/kg suspended in NS with 1.5% glycerinum and 0.5% Tween 80) were freshly prepared. In the systemic cryptococcosis model, Each mouse was inoculated with 2×10^5 CFU of *C. neoformans* cells (0.2 mL, 1×10^6 CFU/mL in saline). Two hours later, all the mice were divided into three groups and injected intraperitoneally with saline, FLC or compound **B10** respectively for five days. The mice were sacrificed and dissected on the 6th day, the brain tissue of each mouse was removed and homogenized in 1 mL of NS. The homogenates were diluted to different concentrations with PBS, and inoculated on Sabouraud's Dextrose Agar (SDA) medium with chloromycetin (100 μg/mL). The number of colonies on each medium plate was counted in order to calculate the fungal burden, and the differences among groups were analyzed by one-way ANOVA. The similar protocol was applied in the systemic candidiasis model, except that 100 mg/kg of cyclophosphamide was injected intraperitoneally as an immunosuppressive agent 24 h before the inoculation. The evaluation of mice survival time was performed according to a similar protocol except that the mice would not be sacrificed on the 6th day.

Cell Cycle Analysis by Flow Cytometry. Cell cycle analysis was performed according to reported protocol[45] with a few modifications. *C. neoformans* cells during the exponential growth period were harvested and re-suspended to 1×10^6 CFU/mL in 5mL of RPMI 1640 medium with different concentrations of FLC and compound **B10**. After being incubated for 24 h at 30°C with constant shaking (200 rpm.), each sample was centrifuged and allowed to stand for 20 h with 70% ethanol. The cells were then stained with 50 μg/mL of propidium iodide for 30 min at 4°C, and sonicated to get separate cell. The data was collected using a FACSCalibur cytometer (Becton, Dickinson, San Jose, CA) and analyzed with Cell Quest 3.0 software.

Transmission Electron Microscopy. Transmission electron microscopy analysis was performed according to the reported protocol.[46] *C. neoformans* (or *C. albicans*) cells were collected after being cultured for 24 h at 30 °C in liquid RPMI 1640 medium with or without FLC and/or compound **B10**, and then washed twice with PBS solution, allowed to stand for 24 h at 4°C in 500 mL of fixative solution (sodium cacodylate buffer, pH 7.2, containing 4% polyoxymethylene). The samples were then washed with saline and allowed to stand for 90 min with 1% phosphotungstic acid. The obtained cells were dehydrated with a graded series of ethanol and embedded with EPON-812. After being double stained with uranium and lead, ultrathin sections were prepared and observed under a transmission electron microscope (HITACHI H-800, Japan) with 5×10^3 magnification.

Real-time RT-PCR. RNA isolation was performed exactly according to the manufacture's protocol (TaKaRa, Biotechnology, Dalian, PR China), and cDNA was

obtained by a reverse transcription kit (TaKaRa) through reverse transcription reaction. Real-time RT-PCR was performed by LightCycler Real-Time PCR system (Roche diagnostics, GmbH Mannheim, Germany), using SYBR Green I.[60] The experimental procedure for thermal cycling consisted of a step at 95 ^{o}C for 30 s, and then 40 cycles of 95 ^{o}C for 5 s, 60 ^{o}C for 34 s, and 95 ^{o}C for 30 s, 60 ^{o}C for 60 s, 95 ^{o}C for 15 s. The fluorescence change of SYBR Green I was monitored in each cycle, and the threshold cycle (C_T) was measured. ACT1 was used as an internal control. Compared with ACT1, relative fold change of the gene expression levels for the cells untreated or treated with FLC alone or FLC and compound **B10**, were calculated using the formula 2DDCT. Real-Time PCR reaction for each cDNA was performed in triplication. Gene-specific primers are shown in Table S2 in Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXXX.

Chemical synthesis and structural characterization of intermediates 4a-4g, 7, 11b and 15a-15d, *in vitro* BBB permeation assay and permeability of compound B10, primers for real-time RT-PCR, HTRF cAMP assay, *in vitro* metabolic stability assay, the evaluation of the production of capsular polysaccharide, sterol composition analysis, the evaluation of the efflux of rhodamine 6G and ^{1}HNMR, ^{13}CNMR, HRMS, HPLC spectra of the representative compounds.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENT

This work was supported by the National Natural Science Foundation of China (Grant 81725020 to C.S.), the Innovation Program of Shanghai Municipal Education Commission (Grant 2019-01-07-00-07-E00073 to C.S.) and Science and Technology Commission of Shanghai Municipality (Grant 17XD1404700 to C. S.).

■ ABBREVIATIONS LIST

IFI, invasive fungal infection; HIV, Human Immunodeficiency Virus; CM, cryptococcal meningitis; FLC, fluconazole; RT-PCR, Reverse Transcription-Polymerase Chain Reaction; TFA, trifluoroacetic acid; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; HATU, 2-(7-azabenzotriazol

-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate; THF, tetrahydrofuran; ACN, acetonitrile; HAL, haloperidol; FICI, fractional inhibitory concentration; MIC, minimal inhibitory concentration; CFU, colony-forming units; *L*-DOPA， levodopa; XTT, 2,3-bis(2-hydroxyethylthio)naphthalene-1,4-dione; TEM, transmission electron microscopy; CYP51, lanosterol 14a- demethylase; cAMP, cyclic Adenosine monophosphate; GLC, glucose; ATP, adenosine triphosphate; HTRF, homogeneous time-resolved fluorescence; RPMI, roswell park memorial institute; PBS, phosphate buffer saline; $T_{1/2}$, half time; BBB, blood-brain barrier; PAMPA, parallel artificial membrane permeation assay; DIC, differential interference contrast; SDA, sabouraud medium agar; DMSO, dimethyl sulfoxide; PI, propidium iodide; NS, normal saline; NADPH, nicotinamide adenine dinucleotide phosphate; CHO, Chinese hamster ovary; ICR, institute of cancer research; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane -sulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; BSA, albumin from bovine serum; GXM, glucuronoxylomannan; GalXM, glucuronoxylomannogalactan; MST, median survival time; YNB, Yeast Nitrogen Base; rpm, revolutions per minute; ddH_2O, double distilled water; *C. alb*., *Candida albicans*; *C. gla*., *Candida glabrata*; *C. neo*., *Cryptococcus neoformans*; *C. tro*., *Candida tropicalis*; *C. par*., *Candida parapsilosis*; *C. kru*., *Candida krusei*.

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