

Novel Carboline Derivatives as Potent Antifungal Lead Compounds: Design, Synthesis, and Biological Evaluation

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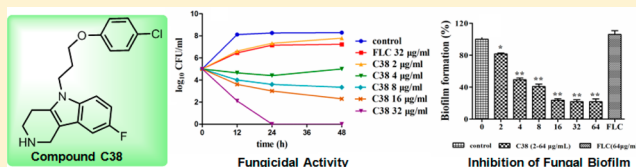
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S Supporting Information

ABSTRACT: A series of novel antifungal carboline derivatives was designed and synthesized, which showed broad-spectrum antifungal activity. Particularly, compound C38 showed comparable in vitro antifungal activity to fluconazole without toxicity to human embryonic lung cells. It also exhibited good fungicidal activity against both fluconazole-sensitive and -resistant *Candida albicans* cells and had potent inhibition activity against *Candida albicans* biofilm formation and hyphal growth. Moreover, C38 showed good synergistic antifungal activity in combination with fluconazole (FLC) against FLC-resistant *Candida species*. Preliminary mechanism studies revealed that C38 might act by inhibiting the synthesis of fungal cell wall.

KEYWORDS: Antifungal lead compounds, carboline derivatives, fungicidal activity



In recent years, the incidence and associated mortality of invasive fungal infections (IFIs) has been increasing dramatically.^{1,2} However, effective and safe antifungal agents are very limited. Clinically available antifungals (Figure 1) for IFIs mainly include: polyenes (e.g., amphotericin B), fluorinated pyrimidines (e.g., 5-fluorocytosine), azoles (e.g., fluconazole and voriconazole), and echinocandins (e.g.,

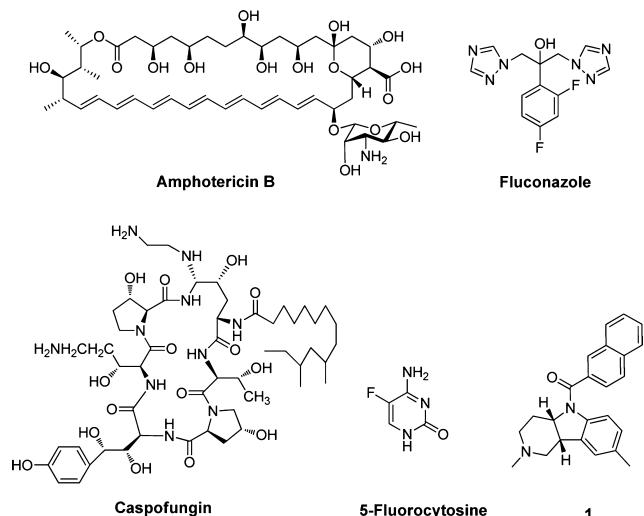


Figure 1. Chemical structures of representative antifungal agents for the treatment of invasive fungal infections and hit compound 1.

casposungin, micafungin, and anidulafungin).^{3,4} Among them, amphotericin B has been used as the gold standard for almost all IFIs for more than 40 years, but severe nephrotoxicity has been associated with this drug.^{5,6} 5-Fluorocytosine is usually used as adjunctive therapy. In combination with amphotericin B, it is effective against a number of mycoses, including some caused by the *cryptococcus spp.*, *dematiaceous* fungi, and some *Candida spp.* The azole antifungals have emerged as front-line drugs in antifungal chemotherapy.^{7,8} Among the azoles, fluconazole plays an important role in treating both superficial and invasive yeast fungal infections due to its favorable pharmacokinetics, excellent activity against *Candida spp.*, and safety profile.^{7,8} However, it is not effective against invasive *aspergillosis* and has suffered severe drug resistance. Echinocandin antifungals show potent activity against *Candida* and *Aspergillus spp.* but are not active in other common and emerging pathogens, such as *Fusarium*, *Scedosporium*, and *Zygomycetes*.⁹ Moreover, resistance to echinocandins has also been reported.¹⁰ Therefore, there is a need to discover and develop antifungal agents with novel chemotype and fungal-specific mode of action.

Continuing our efforts on antifungal drug discovery,^{11–15} we performed a cell-based antifungal screen of an in-house library using the standard NCCLS (National Committee for Clinical Laboratory Standards) protocol. Compound 1 with a β -

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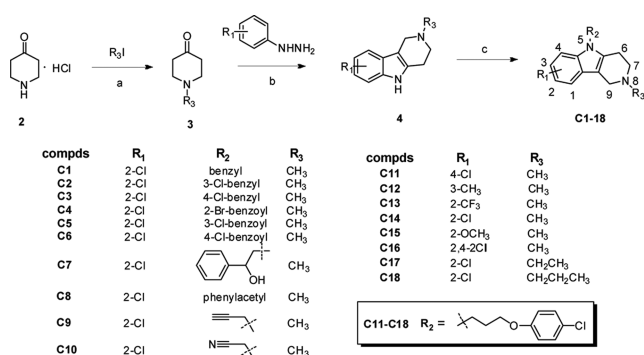
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carboline scaffold was identified as a modest fungal inhibitor, with an MIC₈₀ value of 32 and 8 μg/mL against *Candida albicans* and *Cryptococcus neoformans*, respectively. Because the chemical scaffold of compound **1** differs from that of all reported antifungal agents, it is interesting to investigate its structure–activity relationships (SARs) and discover the novel antifungal lead compound.

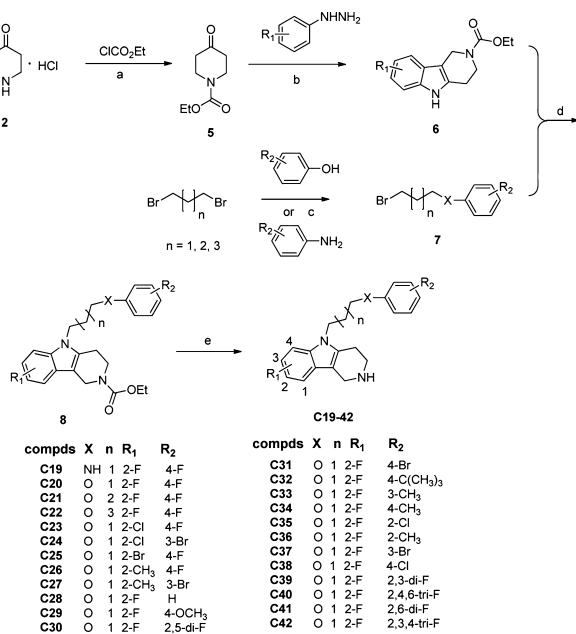
Thus, a series of novel carboline derivatives was designed and synthesized (Schemes 1 and 2). The in vitro antifungal

Scheme 1. Chemical Synthesis of Compounds C1–C18^a



^aReagents and conditions: (a) K₂CO₃, EtOH, reflux, 2 h; (b) 1,4-dioxane, conc. H₂SO₄, 80 °C, 3 h; (c) base (NaH or KOH), solvent (DMF or DMSO), rt or 45 °C.

Scheme 2. Chemical Synthesis of Compounds C19–C42^a



^aReagents and conditions: (a) K₂CO₃, CH₂Cl₂, rt, 6 h; (b) EtOH, reflux, 2 h; (c) K₂CO₃, EtOH, reflux, 4 h; (d) DMSO, KOH, rt, 12 h; (e) EtOH, Claisen hydrolysis, reflux, 3 h.

activities are summarized in Tables 1 and 2. Most of the target compounds showed moderate to good inhibitory activity with broad spectrum. Among them, compound C38 showed potent inhibitory activity against all the tested fungal pathogens (MIC₈₀ range: 1 to 4 μg/mL). Its activity against *C. albicans* (MIC₈₀ = 2 μg/mL), *C. neoformans* (MIC₈₀ = 1 μg/mL), *M. gypseum* (MIC₈₀ = 1 μg/mL), and *C. krusei* (MIC₈₀ = 4 μg/mL) was comparable or superior to FLC. Moreover, the cytotoxicity

Table 1. In Vitro Antifungal Activity of Compounds C1–C18 (MIC₈₀, μg·mL⁻¹)^a

compd	<i>C. alb.</i>	<i>C. par.</i>	<i>C. neo.</i>	<i>C. gla.</i>	<i>A. fum.</i>	<i>T. rub.</i>	<i>M. gyp.</i>
1	32	16	8	>64	>64	16	32
C1	64	64	32	32	64	16	16
C2	64	32	16	16	32	8	16
C3	64	64	16	16	64	64	16
C4	>64	64	>64	>64	>64	>64	>64
C5	>64	64	32	32	>64	>64	>64
C6	>64	64	32	32	>64	32	>64
C7	>64	64	32	32	>64	>64	32
C8	>64	>64	>64	>64	>64	>64	>64
C9	>64	64	32	64	>64	>64	32
C10	>64	>64	64	>64	>64	>64	64
C11	32	32	16	32	>64	>64	16
C12	32	16	8	16	16	8	8
C13	>64	>64	64	64	>64	64	64
C14	64	16	8	16	>64	>64	16
C15	32	32	8	>64	32	8	8
C16	>64	>64	16	>64	>64	>64	16
C17	64	64	16	32	>64	>64	32
C18	>64	64	16	16	>64	>64	16
FLC	0.5	2	2	0.5	>64	4	1

^aAbbreviations and strain numbers: *C. alb.*, *Candida albicans* (SC5314); *C. par.*, *Candida parapsilosis* (ATCC 22019); *C. neo.*, *Cryptococcus neoformans* (32609); *C. gla.*, *Candida glabrata* (537); *A. fum.*, *Aspergillus fumigatus* (07544); *T. rub.*, *Trichophyton rubrum* (Cmccftla); *M. gyp.*, *Microsporium gypseum* (Cmccfmza); FLC, fluconazole.

of C38 against WI38 cell line (human embryonic lung cell) was determined using the standard MTT method. The IC₅₀ value of C38 was larger than 40 μg/mL, indicating that it had selective antifungal activity.

Time-kill testing assay was used to test whether this new type of antifungal compound had fungicidal activity. The blank control and FLC at 32 μg/mL were used as controls. As shown in Figure 2A, compound **1** showed no significant difference compared with FLC against *C. albicans* SC5314 (FLC sensitive, MIC₈₀ = 0.5 μg/mL), indicating that it may only had fungistatic activity, which was similar to that of FLC. The fungicidal activity of C38 was also investigated at the concentration range of 2–32 μg/mL. As shown in Figure 2B, C38 at 2 μg/mL showed no significant difference compared with FLC at 32 μg/mL against the *C. albicans* SC5314 strain. Interestingly, C38 showed fungicidal activity at the concentration of 4 μg/mL. When the concentration was increased to 8 and 16 μg/mL, the fungicidal activity was enhanced. To our delight, under the treatment of C38 at 32 μg/mL for 24 h, density of fungi cells was reduced to zero, whereas FLC had no significant effect at the same concentration. The results confirmed that carboline derivative C38 had excellent fungicidal activity against FLC-sensitive *C. albicans* in a dose-dependent manner.

Importantly, fungal resistance caused by broad use of azoles is becoming a serious problem in recent years.¹⁶ It is highly desirable to find a new type of inhibitors that could be effective against azole-resistant fungal cells. *C. albicans* 103 (FLC resistant, MIC₈₀ > 64 μg/mL) was used to test whether C38 could be effective against FLC-resistant fungi. As shown in Figure 2C, C38 obviously inhibited the growth of *C. albicans* 103 at the concentrations of 4 and 8 μg/mL. When the concentration was increased to 16 and 32 μg/mL, *C. albicans*

Table 2. In Vitro Antifungal Activity of Compounds C19–C42 (MIC₈₀, μg·mL⁻¹)^a

compd	<i>C. neo.</i>	<i>C. alb.</i>	<i>C. par.</i>	<i>C. tro.</i>	<i>C. krusei</i>	<i>T. rub.</i>	<i>M. gyp.</i>
C19	32	8	16	64	16	32	16
C20	8	8	16	16	4	8	8
C21	16	16	16	16	4	16	8
C22	16	16	16	4	4	32	8
C23	4	4	4	4	4	8	4
C24	4	8	16	16	4	16	16
C25	4	4	4	16	4	8	4
C26	16	4	16	64	16	4	4
C27	16	16	16	16	4	16	16
C28	8	8	>64	64	16	8	4
C29	8	8	>64	>64	16	8	4
C30	16	4	64	16	4	8	2
C31	4	8	64	16	4	8	2
C32	8	8	4	4	16	4	4
C33	8	8	4	16	16	4	4
C34	4	8	4	16	4	2	4
C35	16	32	64	64	64	32	16
C36	4	4	16	64	16	4	4
C37	4	2	4	16	4	4	2
C38	1	2	4	4	4	2	1
C39	32	32	4	16	4	32	16
C40	4	8	4	16	4	4	4
C41	8	4	64	16	4	2	1
C42	4	8	64	4	4	8	4
FLC	2	0.5	2	1	4	4	1

^aAbbreviations and strain numbers: *C. alb.*, *Candida albicans* (SC5314); *C. par.*, *Candida parapsilosis* (ATCC 22019); *C. neo.*, *Cryptococcus neoformans* (32609); *C. tro.*, *Candida tropicalis*; *C. krusei*, *Candida krusei* (0710397); *T. rub.*, *Trichophyton rubrum* (Cmccftla); *M. gyp.*, *Microsporium gypseum* (Cmccfmza); FLC, fluconazole.

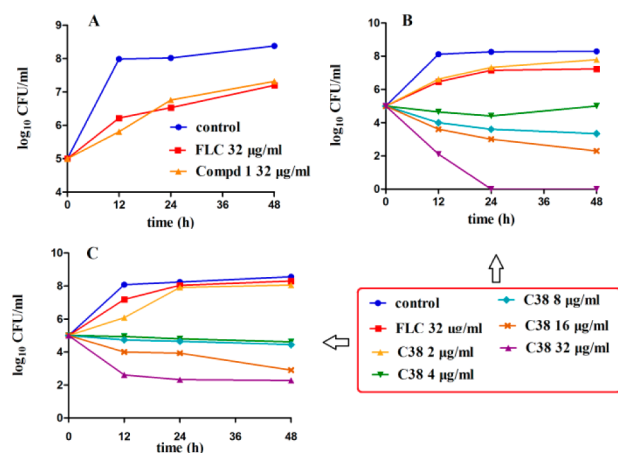


Figure 2. Time-kill curves of *C. albicans* obtained by using an initial inoculum of 10⁵ CFU/mL. CFUs/mL were determined after 0, 12, 24, and 48 h of incubation. (A) *C. albicans* SC5314 (FLC-sensitive, MIC₈₀ = 0.5 μg/mL) was treated/untreated with FLC (32 μg/mL) or lead compound 1 (32 μg/mL). (B) *C. albicans* SC5314 was treated/untreated with FLC (32 μg/mL) or different concentrations of compound C38. (C) *C. albicans* 103 (FLC-resistant, MIC₈₀ > 64 μg/mL) was treated/untreated with FLC (32 μg/mL) or different concentrations of compound C38.

cells were decreased to a larger extent. The results confirmed that compound C38 had potent fungicidal activity against FLC-resistant *C. albicans*.

An important reason for the severe drug resistance is the strong ability of *Candida* cells to form biofilms.^{17,18} More over, biofilms could shield *Candida* cells from attack by the immune system and also block antifungal agents from reaching infected cells.^{19,20} The ability of C38 to inhibit biofilm formation of *C. albicans* SC5314 (FLC sensitive) and *C. albicans* 103 (FLC resistant) was investigated. FLC at 64 μg/mL and the blank control were used as controls. For the strains of *C. albicans* SC5314 and *C. albicans* 103 (Figure 3), the fungi cells treated

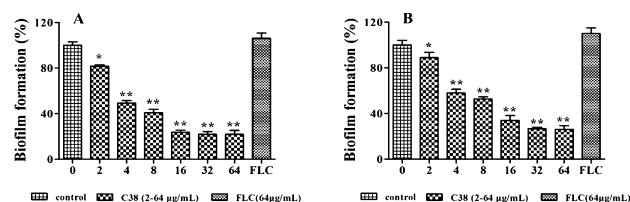


Figure 3. Effects of different concentrations of compound C38 on biofilm formation. *C. albicans* SC5314 (A) and *C. albicans* 103 (B) were used in this assay. Cells were treated with compound C38 (2–64 μg/mL) or FLC (64 μg/mL). Biofilm formation (as measured by XTT reduction) is expressed as a percentage of that of blank control. Results represent the mean ± standard deviations for three independent experiments. Statistical significance among the groups was determined by the analyses of variance (ANOVA). For comparison between two groups, the Student *t* test was used. A *p*-value of less than 0.05 was considered significant. **p* < 0.05 compared to the control group; ***p* < 0.01 compared to the control group.

with 64 μg/mL of FLC showed no inhibitory effect of biofilm formation and had no significant difference compared with the blank control, which was consistent with the previous reports.^{21–23} Interestingly, C38 showed a potent inhibitory effect on biofilm formation. Moreover, the inhibitory effect appeared to be dose-dependent. As shown in Figure 3A, C38 could inhibit >50% of biofilm formation at the concentration of 4 μg/mL. When the concentration was increased to 64 μg/mL, biofilm formation was significantly inhibited (>80%). More importantly, C38 also showed potent inhibitory effect on biofilm formation of the FLC-resistant *C. albicans* 103 strain (Figure 3B). It inhibited biofilm formation by >75% at the concentration of 32 μg/mL. The biofilm inhibition assay confirmed that C38 showed dose-related inhibitory activity on biofilm formation and that it was effective against both FLC-sensitive and FLC-resistant fungi.

A striking feature of *C. albicans* biology is its ability to grow in a variety of morphological forms, such as yeast, pseudohyphae, and hyphae.²⁴ In particular, the hyphae form could promote fungi cells to penetrate human epithelial and endothelial cells during the early stages of infection and cause damage.^{25,26} Moreover, hyphae is essential for pathogenicity by forming biofilms,²⁷ which are highly resistant to standard antifungal treatments. Therefore, the inhibitory ability of C38 on the growth of *C. albicans* hyphae was determined (Figure 4). In the blank control group (Figure 4A), most of the *C. albicans* cells showed elongated hyphae forms. After treated with FLC at 8 μg/mL (Figure 4B), the hyphal growth was not inhibited, and the morphology was similar to that of the blank control. The result was consistent with the fact that FLC was not effective in *C. albicans* hyphal growth.^{28,29} After treated with C38 at 2 μg/mL (Figure 4C), most of the *C. albicans* cells appeared as pseudohyphae form, and the cells were much shorter than the control group. More interestingly, only unicellular budding

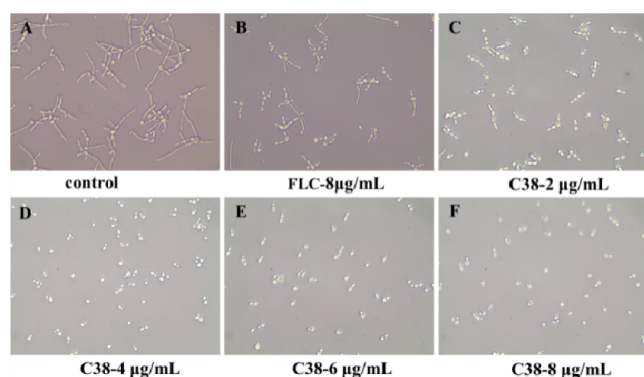


Figure 4. Effects of different concentrations of compound C38 or FLC on *C. albicans* SC5314 hyphal growth. The fungi cells were incubated in RPMI 1640 liquid medium at 37 °C for 16 h in the absence or presence of different concentrations of compound C38 (2 μg/mL to 8 μg/mL) or FLC (8 μg/mL). The hyphal development was viewed by light microscopy at ×400 and photographed.

yeast form was observed after exposure with C38 at 4 μg/mL or higher concentration (Figures 4D–F). In this assay, C38 showed potent inhibitory effect on the *C. albicans* hyphal growth, whereas FLC was ineffective.

The *in vitro* synergistic antifungal activity of compound C38 in combination with FLC was also conducted (Table 3). Four

Table 3. In Vitro Synergistic Antifungal Activities of Compound C38 and FLC (MIC₈₀, μg·mL⁻¹)

clinical isolates	MIC ₈₀ alone		MIC ₈₀ in combination		FICI ^a	mode of interaction
	FLC	C38	FLC	C38		
103	>1024	4	2	1	0.252	syn
100	>1024	4	2	1	0.252	syn
07109	>1024	4	2	1	0.252	syn
805	>1024	4	2	1	0.252	syn

^aSynergy and antagonism were defined by FICI ≤ 0.5 and >4, respectively. Indifferent was defined by 0.5 < FICI ≤ 4.

FLC-resistant *C. albicans* spp. (MIC₈₀ > 1024 μg/mL) were used in this testing. To our delight, C38 alone showed good inhibitory activities against all tested *Candida* species with MIC₈₀ values of 4 μg/mL. More interesting, C38 showed good synergistic activities in combination with FLC. The fractional inhibitory concentration index (FICI) value of C38 was 0.252, indicating C38 exhibited good synergistic activities against tested *Candida* species in combination with FLC. In this assay, C38 not only showed good antifungal activities against FLC-resistant *Candida* species but also showed potent synergistic activities in combination with FLC.

In order to reveal the antifungal mechanism of compound C38, its influence on the morphology of *C. albicans* was monitored by transmission electron microscopy (TEM). The normal *C. albicans* cells and cells treated with FLC at 8 μg/mL for 8 h were used as controls. As shown in Figure 5A, the normal *C. albicans* cell has a uniform central density and an intact cell wall. For the cells treated with FLC (Figure 5B), the cell membrane was damaged obviously, and the shape of the cells became abnormal. These alterations caused by FLC were consistent with the reported results that FLC acts on fungal cell membrane.³⁰ After exposure to C38 (Figure 5C), the outer

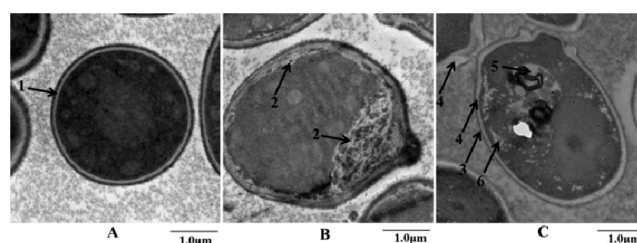


Figure 5. Transmission electron micrographs of *C. albicans* cells under different conditions. The organelles indicated by the arrows are as follows: 1, cell wall; 2, cell membrane; 3, the outer sphere of cell wall; 4, the middle sphere of cell wall; 5, vacuole; 6, reduced cytoplasmic density. The normal *C. albicans* cell (A) shows a uniform central density and a regularly outlined cell wall. The cell treated with FLC at 8 μg/mL for 8 h (B) shows damaged cell membrane and abnormal shape. Picture C shows the cell treated with compound C38 at 8 μg/mL for 8 h. It shows that the outer sphere of cell wall has become thinner and that the middle sphere has become thicker. Moreover, the reduced cytoplasmic density, necrosis, and some abnormal shape of vacuoles could also be observed.

sphere of the cell wall became thinner, and the middle sphere became thicker. No obvious damage was observed on cell membrane. Some abnormal shapes of the vacuoles were also observed. In addition, reduced cytoplasmic density and necrosis could be observed. The TEM results indicated that C38 caused significant damage on *C. albicans* cells. Obvious cell wall change suggests that this new type of antifungal compound may have an effect on some synthetic routes of fungal cell wall.

GC-MS method was used to analyze the change of sterol composition in *C. albicans* cells, which could reflect the effect on *C. albicans* membrane. The assay has been successfully used in studying the mechanism of fungal resistance^{31,32} and the influence of inhibitors on sterol biosynthesis pathway.³³ Cholesterol was added as internal standard and the results were shown in Table 4. In the blank control group, ergosterol

Table 4. Relative Sterol Composition of *C. albicans* SC5314 after 24 h of Incubation

sterol	% of total sterols (<i>Candida albicans</i> SC5314) ^a		
	control ^b	C38 ^c	FLC ^d
ergosterol	90.0	40.2	0.6
obtusifoliol	0.0	3.7	6.5
lanosterol	1.5	12.2	5.9
eburicol	0.0	12.4	82.2
unidentified	8.5	31.5	4.8

^aSterol proportions varied by less than 10% in three experiments.

^bControl (no drug). ^cTreated with compound C38 at 8 μg/mL.

^dTreated with FLC at 8 μg/mL. The data was the average of three independent experiments.

and lanosterol comprised 90.0% and 1.5% of the total sterol fraction, respectively. Other 14-methylated sterols such as obtusifoliol and eburicol were not observed. When the cells were treated with FLC at 8 μg/mL for 24 h, the content of eburicol was increased up to 82.2% of the total sterol fraction. Other 14-methylated sterols (lanosterol and obtusifoliol) also increased obviously. In contrast, ergosterol content was reduced to lower than 1% in FLC treated group. These alternations were caused by competitive inhibition of CYP51 by FLC and consistent with the reported results.^{31,32} Interestingly, treatment with C38 also resulted in a significant reduction of

ergosterol content (reduced to 40%) and an obvious accumulation of eburicol and lanosterol precursors (Table 4). However, the sterol composition change was not as obvious as FLC induced effects. The level of ergosterol (about 40% of the total sterol fraction) may not induce obvious change of fungal membrane structure, which was consistent with the results that no obvious damage was observed on cell membrane in the TEM testing. The GC-MS results infer that this new type of inhibitor might have a different mode of action with FLC.

Cytochrome P450 (CYP) enzymes play an important role in the metabolic clearance of the vast majority of prescribed drugs. The assessment of CYP enzymes inhibition or inactivation remains an important role in overall drugs' safety assessment. Clinical drug–drug interactions (DDI) caused by inhibiting these CYP enzymes can result in dangerous side effects, caused by reduced clearance/increased exposure of a second drug.³⁴ Thus, the DDI potential for compound C38 was investigated in vitro using four major human CYP isoforms (2C19, 2C9, 3A4-M, and 3A4-T). As shown in Table 5, compound C38 had weak

Table 5. In Vitro CYP Inhibition Assessment of Compound C38

compd	isoenzyme	% inhibition			IC ₅₀ (μM)	potential inhibition ^a
		25 μM	2.5 μM	0.25 μM		
C38	2C19	33	5	4	>25	low
C38	2C9	36	8	–2	>25	low
C38	3A4-M	–35	–10	1	50	low
C38	3A4-T	50	10	5	25	low

^aIC₅₀ > 10 μM, CYP inhibition low; 10 μM > IC₅₀ > 3 μM, CYP inhibition moderate; 3 μM > IC₅₀, CYP inhibition high.³⁵

inhibitory activity against 2C19, 2C9, 3A4-M, and 3A4-T with IC₅₀ values of ≥25 μM, indicating it had low potential to cause DDI³⁵ (see Supporting Information for data of positive drugs).

In summary, a series of novel antifungal carboline derivatives was designed and synthesized. The most active compound C38 showed several advantages as a promising antifungal lead: (1) it showed broad-spectrum antifungal activity, comparable to FLC; (2) it revealed potent fungicidal activity against both FLC-sensitive and FLC-resistant *C. albicans* cells, whereas FLC only showed fungistatic activity; (3) it was a good inhibitor of *C. albicans* biofilm formation and hyphal growth, suggesting its potential to overcome FLC-related drug resistance; (4) it showed good synergistic antifungal activities in combination with FLC against FLC-resistant *Candida species*; (5) it might have a new mode of action resulting from inhibiting the synthetic route of fungal cell wall; (6) Preliminary CYP enzymes inhibition assay showed C38 had weak inhibition against tested human CYP isoforms, indicating it had low potential to cause DDI. Further structural optimization and antifungal mechanism of the carboline derivatives are in progress.

■ ASSOCIATED CONTENT

⑤ Supporting Information

Structure–activity relationships; chemical synthesis and structural characterization of the target compounds; protocols of biological assays. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

IFI, Invasive fungal infection; AmB, amphotericin B; FLC, fluconazole; SAR, structure–activity relationship; FICI, fractional inhibitory concentration index; CYP, cytochrome P450; DDI, drug–drug interactions; TEM, transmission electron microscopy

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