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Design, synthesis, and evaluation of caffeic acid amides as synergists to sensitize fluconazole-resistant *Candida albicans* to fluconazole

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ABSTRACT

A series of caffeic acid amides were designed, synthesized, and their synergistic activity with fluconazole against fluconazole-resistant *Candida albicans* was evaluated in vitro. The title caffeic acid amides **3**–**30** except **26** exhibited potent activity, and the subsequent SAR study was conducted. Compound **3**, **5**, **21**, and **34c**, at a concentration of 1.0 μg/ml, decreased the MIC_{80} of fluconazole from 128.0 μg/ml to 1.0–0.5 μg/ml against the fluconazole-resistant *C. albicans*. This result suggests that the caffeic acid amides, as synergists, can sensitize drug-resistant fungi to fluconazole. The SAR study indicated that the dihydroxyl groups and the amido groups linking to phenyl or heterocyclic rings are the important pharmacophores of the caffeic acid amides.

Fluconazole is the most commonly used drug to treat *Candida albicans* (*C. albicans*) in the prophylaxis and therapy, however, widespread and repeated use of fluconazole resulted in resistance to or failure of fluconazole therapy.[1] To combat the fluconazole-resistant *C. albicans*, much attention has been paying to the synergism of fluconazole with other agents, as synergists, which can significantly sensitize fungi towards fluconazole,[2,3] for instance, tetracyclic indoles,[4] piperazinyl quinolines,[5] amiodarone,[6] allicin,[7] sulfated Sterols.[8]

Since our previous study indicated that berberine **1** has potent synergistic activity with fluconazole against fluconazole-resistant *C. albicans*,[9] we have been focusing on the structure modification and deconstruction of berberine, which led us to reconstruct a new active scaffold **2**.[10] Inspired by scaffold hopping from the structure of **1**–**2**, we designed and prepared caffeic acid amides **3**–**13**, as shown in Fig. 1, and their synergistic activity with fluconazole against fluconazole-resistant *C. albicans* was evaluated in our lab. As we expected, most of them exhibited potent activity. Herein we report the result and the SAR is investigated and discussed.

Caffeic acid amides **3**–**30** were synthesized by coupling caffeic acid with a series of amines using dicyclohexylcarbodiimide (DCC) as coupling reagent, as shown in Scheme 1. Compounds **16**, **18**, **22**, **27**–**30** are novel and characterized by H NMR and MS.

A series of amines reacted with (*E*)-3-(3,4-dimethoxyphenyl) acrylic acid, and cinnamic acid in the presence of oxalyl chloride and TEA in dichloromethane to give compounds **31a**–**c**, and **32a**–**i**, respectively, as shown in Scheme 2. Compounds **31a**–**c** were treated with Lawesson's Reagent in toluene under reflux to give **33a**–**c**,[11] which were then demethylated with boron tribromide to afford the corresponding phenol **34a**–**c**.[12] Compounds **32h**, **33a**–**c**, **34a**–**c** are novel and characterized by H NMR and MS.

The in vitro synergistic antifungal activities of the title compounds were tested using the microbroth dilution method according to the standards of the Clinical and Laboratory Standards Institute, USA.[13] The MIC_{80} of fluconazole against the fluconazole-resistant *C. albicans* (clinical isolate 103) was determined to be 128.0 μg/ml, while the MIC_{80} values of each title compound used alone, and combined with fluconazole (8.0 μg/ml), were determined as shown in Table 1. Furthermore, the fractional inhibitory concentration index (FICI) of each agent was calculated by summing up the ratios of the MIC_{80} (with FLC)/MIC_{80} (used alone). The interaction modes, synergistic or indifferent, were defined according to FICI values of $\leqslant$0.5 or >0.5, respectively.[9]

At the beginning of this study, caffeic acid anilides **3**–**13** were designed through scaffold hopping based on active compounds **2** and berberine. As shown in Table 1, all the caffeic acid anilides **3**–**13**, at concentrations ranging from 0.5 to 8.0 μg/ml, decreased the MIC_{80} of fluconazole from 128.0 to 8.0 μg/ml. Their FICI values range from 0.070–0.125, which indicated that they have potent synergistic activity with fluconazole. However, the MIC_{80} (used

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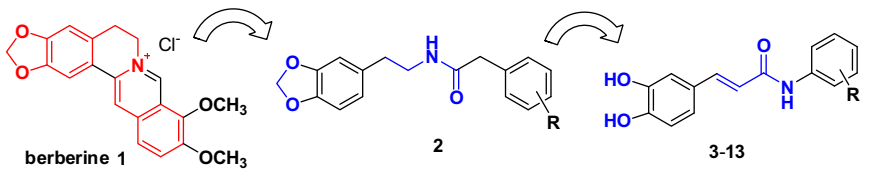
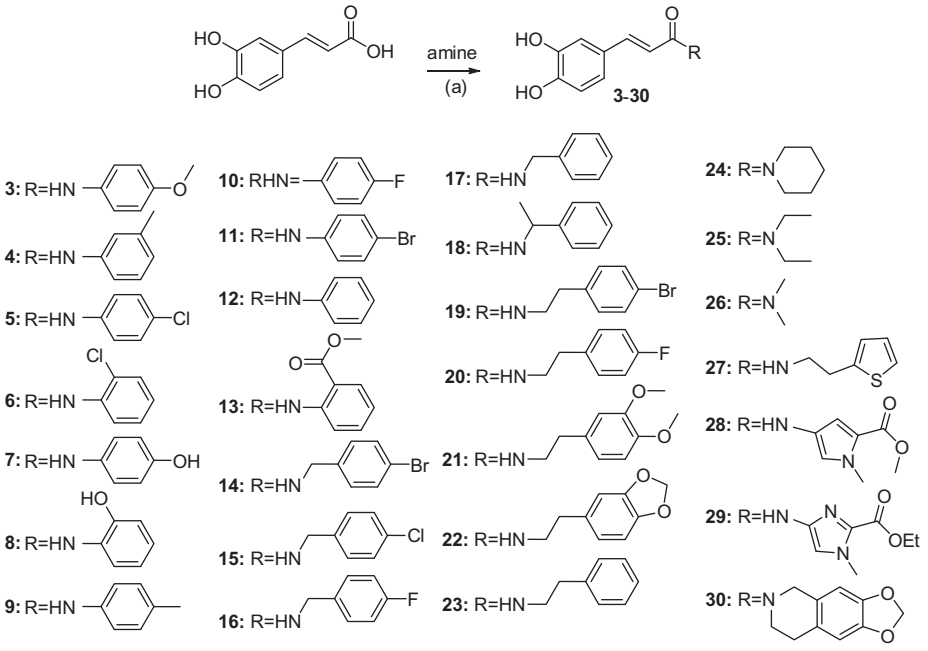
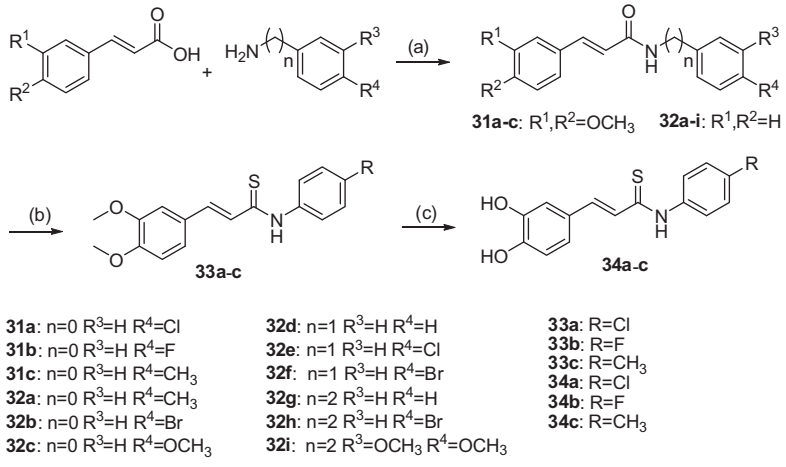


Figure 1. Scaffold hopping design.



Scheme 1. Reagents and conditions: (a) DCC, THF, refluxing, 3–6 h.



Scheme 2. Reagents and conditions: (a) Oxalyl chloride, TEA, CH_2Cl_2, rt, 3–6 h; (b) Lawesson's reagent, toluene, refluxing; (c) BBr_3, CH_2Cl_2, 0 °C.

alone) of the title compounds are >64.0 or 64.0 μg/ml, which suggests that they have no antifungal activity themselves, but they can sensitize the fluconazole-resistant *C. albicans* to fluconazole. Moreover, the anilino moiety in compounds **3**–**13** was replaced: (1) by benzyl and phenylethyl amino groups in compounds **14**–**23**; (2) by alkyl amino groups in compound **24**–**26**; (3) by heterocyclic or heterocyclic alkyl amino groups in compounds **27**–**30**; respectively. All the above-mentioned analogues, except compound **26**, showed good activity with MIC_{80} (with FLC) value ranging from 0.5 to 4.0 μg/ml. However, the MIC_{80} values of caffeic acid, used alone and with fluconazole, were determined to be >64.0 μg/ml, which indicates it has neither antifungal nor synergistic activity with fluconazole. The results suggested the amide moieties are important to their activity.

The MIC_{80} values (with FLC) of compounds **31a**–**c**, **32a**–**i**, and **33a**–**c** are all >64.0 μg/ml, and their FICI are all >0.5, thus indicates they totally lost the synergistic activity. The key point in their structures is that the dihydroxyl groups of caffeic acid moiety were

Table 1
The MIC_{80}, FICI and interaction mode of the title compounds

Compound	MIC_{80} (μg/ml)		FICI	Mode of interaction
	Alone	With FLC[a]		
3	64.0	0.5	0.070	Synergy
4	64.0	0.5	0.070	Synergy
5	64.0	0.5	0.070	Synergy
6	>64.0	4.0	0.094	Synergy
7	>64.0	1.0	0.070	Synergy
8	>64.0	8.0	0.125	Synergy
9	64.0	1.0	0.078	Synergy
10	>64.0	1.0	0.070	Synergy
11	64.0	1.0	0.078	Synergy
12	>64.0	4.0	0.094	Synergy
13	>64.0	4.0	0.094	Synergy
14	>64.0	0.5	0.066	Synergy
15	>64.0	1.0	0.070	Synergy
16	>64.0	1.0	0.070	Synergy
17	>64.0	4.0	0.094	Synergy
18	>64.0	1.0	0.070	Synergy
19	>64.0	1.0	0.070	Synergy
20	>64.0	1.0	0.070	Synergy
21	>64.0	1.0	0.070	Synergy
22	64.0	1.0	0.078	Synergy
23	>64.0	4.0	0.094	Synergy
24	>64.0	4.0	0.094	Synergy
25	>64.0	4.0	0.094	Synergy
26	>64.0	>64.0	>0.5	Indifferent
27	>64.0	4.0	0.094	Synergy
28	64.0	4.0	0.125	Synergy
29	>64.0	4.0	0.094	Synergy
30	64.0	4.0	0.125	Synergy
31a	>64.0	>64.0	>0.5	Indifferent
31b	>64.0	>64.0	>0.5	Indifferent
31c	>64.0	>64.0	>0.5	Indifferent
32a	>64.0	>64.0	>0.5	Indifferent
32b	>64.0	>64.0	>0.5	Indifferent
32c	>64.0	>64.0	>0.5	Indifferent
32d	>64.0	>64.0	>0.5	Indifferent
32e	>64.0	>64.0	>0.5	Indifferent
32f	>64.0	>64.0	>0.5	Indifferent
32g	>64.0	>64.0	>0.5	Indifferent
32h	>64.0	>64.0	>0.5	Indifferent
32i	>64.0	>64.0	>0.5	Indifferent
33a	>64.0	>64.0	>0.5	Indifferent
33b	>64.0	>64.0	>0.5	Indifferent
33c	>64.0	>64.0	>0.5	Indifferent
34a	>64.0	1.0	0.070	Synergy
34b	>64.0	1.0	0.070	Synergy
34c	>64.0	0.5	0.066	Synergy
Caffeic acid	>64.0	>64.0	>0.5	Indifferent
Berberine	32.0	1.0	0.094	Synergy

[a] The concentration of fluconazole (FLC) is 8.0 μg/ml, and the MIC_{80} of fluconazole used alone is 128.0 μg/ml.

replaced by methoxyl groups or hydrogen atoms. Neither lengthening the amide chain by one or two methylene groups in **32d–i**, nor replacing oxygen atom by sulfur atom in the amido bond of **33a–c**, could recover their activity at all. However, compounds **34a–c**, afforded by demethylation of **33a–c**, exhibited potent activity. Moreover, replacing the oxygen atom of amido bond with sulfur atom led to hardly no change in synergic activity, since compounds **5**, **10**, and **9** showed comparable MIC_{80} values (with FLC) to that of **34a**, **34b**, and **34c**, both ranging from 0.5 μg/ml to 1 μg/ml.

In summary, SAR study indicates that both the dihydroxyl groups in caffeic acid moiety and the amide moiety are important pharmacophores of the caffeic acid amides for their synergisitic activity with fluconazole against fluconazole-resistant *C. albicans*. This result is consistent to our previous SAR study on compounds **2**.[10]

For the best combination concentrations of caffeic acid amides and fluconazole, the active compounds **3**, **5**, **21**, and **34c** were subjected to checkerboard microdilution assay, as described in Supporting information. Four MIC_{80} values and FICI values were obtained for each compound as shown in Table 2. The MIC_{80} values of each compound and fluconazole, which gave the lowest FICI, are their best combination concentrations. The lowest FICI values of compound **3**, **5**, **21**, and **34c** are 0.023, 0.016, 0.016, and 0.012 respectively, meanwhile their best MIC_{80} values, with that of fluconazole in the parentheses, are 1.0(1.0), 1.0(1.0), 1.0(1.0) and 1.0(0.5) μg/ml. This result means that the tested compounds, at a concentration of 1.0 μg/ml, can decrease the MIC_{80} of fluconazole from 128.0 to 1.0–0.5 μg/ml against the fluconazole-resistant *C. albicans*. Berberine, which is found to be one of the strongest synergist of fluconazole in our lab, was set to be positive control. The lowest FICI of berberine is 0.035. By comparison of the lowest FICI values, it can be seen that compounds **3**, **5**, **21**, and **34c** showed higher synergistic activity than berberine.

Table 2
Checkerboard microdilution assay of the title compounds and fluconazole

Compound	MIC_{80} (μg/ml)[a]	MIC_{80} (with FLC, μg/ml)[b]	FICI	Mode of interaction
3	64.0	8.0(0.5)	0.129	Synergy
		4.0(0.5)	0.066	Synergy
		2.0(0.5)	0.035	Synergy
		1.0(1.0)	0.023	Synergy
5	>64.0	8.0(0.5)	0.066	Synergy
		4.0(0.5)	0.035	Synergy
		2.0(0.5)	0.020	Synergy
		1.0(1.0)	0.016	Synergy
21	>64.0	8.0(1.0)	0.070	Synergy
		4.0(0.5)	0.035	Synergy
		2.0(1.0)	0.023	Synergy
		1.0(1.0)	0.016	Synergy
34c	>64.0	8.0(0.5)	0.066	Synergy
		4.0(0.5)	0.035	Synergy
		2.0(0.5)	0.020	Synergy
		1.0(0.5)	0.012	Synergy
Berberine	32.0	8.0(0.25)	0.252	Synergy
		4.0(0.5)	0.129	Synergy
		2.0(0.5)	0.066	Synergy
		1.0(0.5)	0.035	Synergy

[a] The fluconazole-resistant *C. albicans* isolate 103.
[b] The corresponding MIC_{80} of fluconazole is in parentheses.

Natural products bearing caffeoyl group have been reported to have antifungal and antibacterial activity.[14,15] Fu et al.[16] reported antifungal activity of caffeic acid amides against drug-sensitive *C. albicans*. In their report, compounds **4**, **5**, and **9–12**, used alone, showed no antifungal activity (MIC >50.0 μg/ml) and compound **3** and **6** showed weak antifungal activity (MIC = 42.8 μg/ml). Their research result is mostly consistent to ours (MIC_{80} >64.0 μg/ml), although the tested *C. albicans* in our research is fluconazole-resistant clinical isolates. However, we have been investigating their antifungal mechanism and target based on our studies on Reactive oxygen species (ROS) and efflux in *C. albicans*, but got no clear results so far. Sandai group reported caffeic acid can inhibit the isocitrate lyase (ICL1) involving the glyoxylate cycle in *C. albicans*.[17] Ma et al. reported their caffeic acid amides inhibited 1,3-β-glucan synthase of *C. albicans*,[18] which may be helpful for us to investigate their mechanism against drug-resistant fungi. Therefore, these finds would provide caffeic acid amides a new kind of chemosensitizers of fluconazole against fluconazole-resistant *C. albicans*.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.11.022.

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