DOI: 10.1002/cmdc.201300332

Structural Optimization of Berberine as a Synergist to Restore Antifungal Activity of Fluconazole against Drug-Resistant *Candida albicans*

Hong Liu,[b] Liang Wang,[a] Yan Li,[a] Jiang Liu,[d] Maomao An,[c] Shaolong Zhu,[a] Yongbing Cao,[a] Zhihui Jiang,[a] Mingzhu Zhao,[a] Zhan Cai,[a] Li Dai,[a] Tingjunhong Ni,[a] Wei Liu,[c] Simin Chen,[c] Changqing Wei,[a] Chengxu Zang,[a] Shujuan Tian,[a] Jingyu Yang,[d] Chunfu Wu,[d] Dazhi Zhang,*[a] Hua Liu,*[b] and Yuanying Jiang*[a]

We have conducted systematic structural modification, deconstruction, and reconstruction of the berberine core with the aim of lowering its cytotoxicity, investigating its pharmacophore, and ultimately, seeking novel synergistic agents to restore the effectiveness of fluconazole against fluconazole-resistant *Candida albicans*. A structure–activity relationship study of 95 analogues led us to identify the novel scaffold of *N*-(2-(benzo[*d*][1,3]dioxol-5-yl)ethyl)-2-(substituted phenyl)acetamides **7a–l**, which exhibited remarkable levels of in vitro synergistic antifungal activity. Compound **7d** (*N*-(2-(benzo[*d*]-[1,3]dioxol-5-yl)ethyl)-2-(2-fluorophenyl)acetamide) significantly decreased the MIC_{80} values of fluconazole from 128.0 μg mL^{-1} to 0.5 μg mL^{-1} against fluconazole-resistant *C. albicans* and exhibited much lower levels of cytotoxicity than berberine toward human umbilical vein endothelial cells.

Introduction

Invasive fungal infections cause significant levels of morbidity and mortality in immunocompromised patients, including those with AIDS, undergoing cancer chemotherapy, or having undergone organ transplantation.[1,2] *Candida albicans* is a particularly common pathogen that is capable of causing life-threatening infections in these patient populations.[2] Fluconazole, originally developed in the early 1990s, is the frontline treatment for systematic *C. albicans* infections.[3] Unfortunately, however, the effective use of fluconazole has been limited significantly by the development of azole resistance, which has been attributed to the failure of antifungal treatments in the clinic.[4–6] One promising approach aimed at overcoming azole resistance has been reported that involves sensitizing *C. albicans* toward fluconazole with small molecules, including piperazinyl quinolines,[7] amiodarone,[8] tacrolimus,[9] honokiol,[10] and Allicin.[11]

The recent screening of a natural product library in our research group revealed berberine (**1**) as the most active synergist in a series of antifungal agents, including fluconazole,[12] ketoconazole, amphotericin B, and miconazole. As a single agent, berberine showed weak antifungal activity (MIC_{80}: 32.0 μg mL^{-1}) against the fluconazole-resistant isolates tested, whereas the MIC_{80} value of fluconazole against the same isolates was 128.0 μg mL^{-1}. When the activity of the two agents in combination was evaluated, the MIC_{80} value of berberine was markedly decreased to 1.0 μg mL^{-1}, and the MIC_{80} value of fluconazole was also decreased to a range of $\leq$0.125–2.0 μg mL^{-1}. Moreover, the occurrence of similar synergistic effects was also reported against disseminated candidiasis in vivo for the combination of berberine with amphotericin B.[13] These synergistic effects indicated the potential for berberine to be used as an antidrug-resistance agent.

Berberine itself does not represent a good starting point for the development of a synergist to be used in combination with antifungal agents because of its poor properties, including its poor solubility,[14] poor rate of absorption,[15] low level of bioavailability in humans,[16] and high toxicity.[17,18] Furthermore, the log *P* value of berberine was determined to be −1.5.[14] We decided to carry out a series of structural modifications, deconstruction, and reconstruction of the berberine core, according to the strategy shown in Figure 1. Pleasingly, the reconstructed compounds **7a–i** restored the effectiveness of fluconazole

[a] L. Wang,[+] Y. Li,[+] S. Zhu, Y. Cao, Z. Jiang, M. Zhao, Z. Cai, L. Dai, T. Ni, C. Wei, C. Zang, S. Tian, Prof. Dr. D. Zhang, Prof. Y. Jiang
School of Pharmacy, Second Military Medical University
325 Guohe Road, Shanghai 200433 (China)
E-mail: zdzhang_yjhx@smmu.edu.cn
jiangyy@smmu.edu.cn

[b] H. Liu,[+] Prof. Dr. H. Liu
School of Pharmacy, Jiangxi University of Traditional Chinese Medicine
18 Yunwan Road, Nanchang, Jiangxi 330004 (China)
E-mail: winner@163.com

[c] M. An, W. Liu, S. Chen
Department of Pharmacology, Tongji University School of Medicine
1239 Siping Road, Shanghai 200092 (China)

[d] J. Liu, Prof. J. Yang, Prof. Dr. C. Wu
School of Life Science and Biopharmaceutics
Shenyang Pharmaceutical University
103 Wenhua Road, Shenyang, Liaoning 110016 (China)

[+] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201300332.

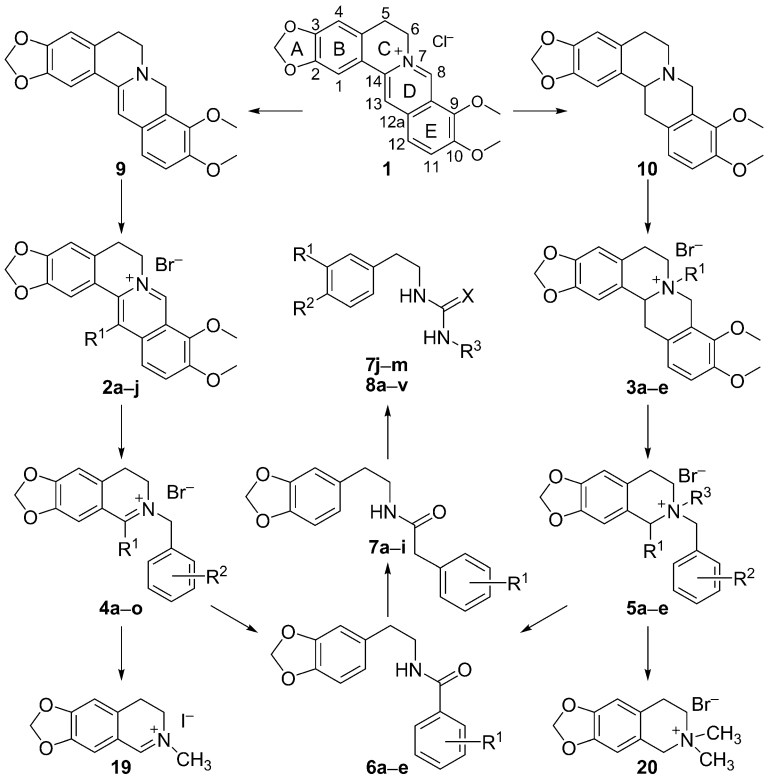


Figure 1. General structures of the designed analogues of berberine.

against drug-resistant *C. albicans*, with some of the synthesized compounds exhibiting similar levels of activity to berberine. Furthermore, the cytotoxicities of compounds **7b**, **7d**, and **7e** were much lower than that of berberine.

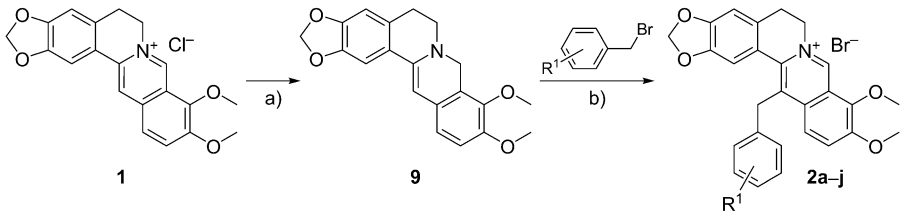
Results

Chemistry

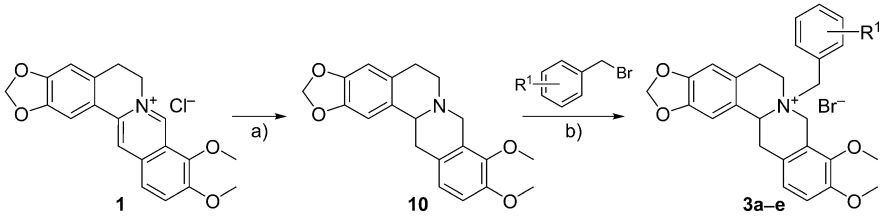
The structural modification process began with reduction of the iminium ion in berberine. Berberine (**1**) was treated with potassium carbonate, and the resulting mixture was subsequently reacted with sodium borohydride in a 5% aqueous sodium hydroxide solution to give dihydroberberine **9**. In a separate experiment, berberine was treated with sodium borohydride in methanol at reflux to give tetrahydroberberine **10** in good yield. Compounds **2a–j** were obtained by holding a mixture of **9** at reflux with the appropriate alkyl halide in acetonitrile (Scheme 1).[19] In the alkylation reactions of tetrahydroberberine **10**, the designed quaternary ammonium salts **3a–e** were obtained by holding a mixture of **10** at reflux with the corresponding substituted benzyl halides in acetonitrile (Scheme 2).[19]

The 3,4-dihydroisoquinolin-2-iums **4a–o** were synthesized in three steps. The initial amidation of 2-(benzo[*d*][1,3]dioxol-5-yl)ethanamine (**15**) with a series of carboxylic acids gave the corresponding intermediates **11a–d**, which underwent the Bischler–Napieralski reaction in the presence of phosphoryl chloride in toluene at reflux to afford **12a–d**.[20] In the final stage, the nitrogen atoms in **12a–d** were alkylated with benzyl halides to give **4a–o** (Scheme 3).[21] Compound **19**, shown in Figure 1, was synthesized by holding a solution of **12a** and methyl iodide at reflux in acetonitrile.

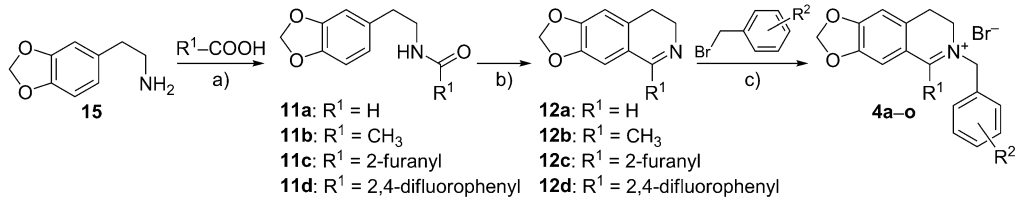
The 1,2,3,4-tetrahydroisoquinolin-2-iums **5a–e** were synthesized according to a three-step procedure. Starting material **15** was treated with a variety of different aldehydes under Pictet–Spengler reaction conditions to afford compounds **13a** and **b**,[22,23] the nitrogen atoms of which were alkylated via substitution with alkyl halides to give corresponding alkylated derivatives **14a–d**.[22] Further alkylation of compounds **14a–d** with benzyl bromides gave compounds **5a–e** (Scheme 4). Compound **20**, shown in Figure 1,



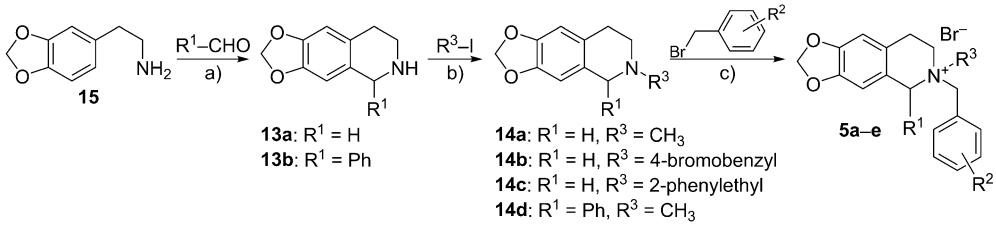
Scheme 1. Synthesis of derivatives **2a–j**. *Reagents and conditions*: a) $NaBH_4$, MeOH, K_2CO_3, 5% $NaOH_{(aq)}$, RT, 1 h; b) CH_3CN, KI, reflux, 4 h.



Scheme 2. Synthesis of derivatives **3a–e**. *Reagents and conditions*: a) $NaBH_4$, MeOH, reflux, 1 h; b) CH_3CN, reflux, 6 h.



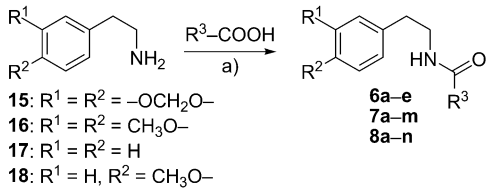
Scheme 3. Synthesis of derivatives **4a–o**. *Reagents and conditions*: a) HOBt, DCC, THF, RT, 12 h; b) $POCl_3$, toluene, reflux, 8–12 h; c) CH_3CN, KI, reflux, 6 h.



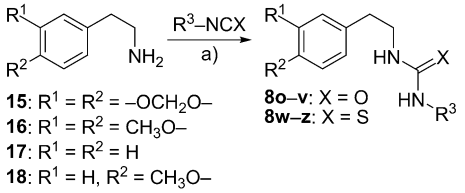
Scheme 4. Synthesis of derivatives **5a–e**. *Reagents and conditions*: a) TFA, reflux, 12–24 h, NaOH; b) CH_2Cl_2, K_2CO_3, reflux, 8–12 h; c) CH_2Cl_2, KI, reflux, 8 h.

was synthesized by holding a solution of **14a** at reflux with methyl iodide in acetonitrile.

A series of 2-(substituted phenyl)ethylamines, including **15**, **16**, **17**, and **18**, were reacted as starting materials with a series of different carboxylic acids,[24] isocyanates,[25] and isothiocyanates[25] to give the corresponding amides **6a–e**, **7a–m**, and **8a–n**, ureas **8o–v**, and thioureas **8w–z**, respectively, as shown in Schemes 5 and 6.



Scheme 5. Synthesis of amide derivatives. *Reagents and conditions*: a) HOBt, DCC, THF, RT, 6–12 h.



Scheme 6. Synthesis of urea and thiourea derivatives. *Reagents and conditions*: a) for ureas: THF, RT, 6–12 h; for thioureas: THF, RT, 4–6 h.

Biological evaluation

The in vitro synergistic antifungal activities of the newly synthesized berberine analogues were tested using the microbroth dilution method, according to the standards of the Clinical and Laboratory Standards Institute, USA (formerly the National Committee for Clinical Laboratory Standards).[26] When fluconazole-resistant *C. albicans* was treated with fluconazole or berberine analogues the individual MIC_{80} values were determined and defined as the lowest concentration of the agents that inhibited growth by 80%. When *C. albicans* was treated with a combination of fluconazole and berberine analogues, the interaction MIC_{80} value of each agent was obtained. Furthermore, the fractional inhibitory concentration index (FICI) of each agent was calculated by determining the ratio of the interaction MIC_{80}/individual MIC_{80}. The interaction modes, either synergistic or indifferent, were defined according to FICI values of ≤ 0.5 or > 0.5, respectively.[27] In this assay, the final concentration of fluconazole (FLC, in Tables 1 and 2) in each well was fixed at a single value (16.0 $\mu g\,mL^{-1}$), whereas the final concentration of the berberine analogues was set at a range of 0.13–64.0 $\mu g\,mL^{-1}$ in a double dilution series. Four clinical isolates of fluconazole-resistant *C. albicans* (with MIC_{80} values determined as 128.0 $\mu g\,mL^{-1}$) were used in this study. In this paper, we have chosen to only report the results obtained with *C. albicans* 103, which were found to be consistent with that of the other three isolates (*C. albicans* 100, J28, and 953).

As shown in Table 1, dihydroberberine **9** and tetrahydroberberine **10** did not show synergistic activity in combination with fluconazole (16.0 $\mu g\,mL^{-1}$). However, compounds **2a-j**, which were derived from **9**, showed good synergistic activity. The interaction MIC_{80} values of compounds **2a–j** were in the range of 0.25–8.0 $\mu g\,mL^{-1}$, and the FICI values of compounds **2a**, **2b**, **2c**, **2d**, **2e**, **2f**, and **2j** in particular were in the range of 0.129–0.141, indicating that these compounds exhibited a higher level of synergistic activity than berberine. On the basis of these results, it was concluded that the introduction of a benzyl group at the C13 position could improve the synergistic activity of berberine. One question raised by this result, however, was whether functionality at the C13 position was critical to the activity of berberine, and if so, whether the introduction of a bulky benzyl group at this position would affect the ability of the derivatives to bind to the target and consequently lead to a decrease in their activity. To address these issues, compounds **4a–o** were designed and prepared as deconstructed berberine analogues without the C13 moiety.

Table 1. Structures and interaction modes of the title compounds and their MIC_{80} and FICI values.

Compd	R^1	R^2	R^3	MIC_{80} [$\mu g\,mL^{-1}$]	MIC_{80} with FLC[a]	FICI	Mode of Interaction
1	–	–	–	32.0	1.0	0.156	synergy
9	–	–	–	>64.0	>64.0	>0.50	indifferent
10	–	–	–	>64.0	>64.0	>0.50	indifferent
2a	*o*-Br	–	–	32.0	0.25	0.133	synergy
2b	*m*-Br	–	–	32.0	0.5	0.141	synergy
2c	*p*-Br	–	–	64.0	1.0	0.141	synergy
2d	*o*-F	–	–	64.0	0.25	0.129	synergy
2e	*p*-OMe	–	–	32.0	2.0	0.141	synergy
2f	*p*-F	–	–	64.0	1.0	0.141	synergy
2g	*m*-NO_2	–	–	>64.0	8.0	0.187	synergy
2h	*m*-Me	–	–	32.0	1.0	0.156	synergy
2i	*o*-NO_2	–	–	32.0	1.0	0.156	synergy
2j	*m*-F	–	–	>64.0	1.0	0.133	synergy
3a	*o*-NO_2	–	–	>64.0	32.0	0.375	synergy
3b	*p*-NO_2	–	–	>64.0	32.0	0.375	synergy
3c	*m*-F	–	–	>64.0	16.0	0.250	synergy
3d	*o*-Cl	–	–	>64.0	4.0	0.156	synergy
3e	*o*-Me	–	–	>64.0	4.0	0.156	synergy
4a	2-furanyl	4-*t*Bu	–	>64.0	4.0	0.156	synergy
4b	2,4-difluorophenyl	4-*t*Bu	–	64.0	4.0	0.188	synergy
4c	H	4-*t*Bu	–	>64.0	8.0	0.188	synergy
4d	Me	4-*t*Bu	–	>64.0	16.0	0.250	synergy
4e	H	2,3-dimethoxy	–	>64.0	16.0	0.250	synergy
4f	Me	2,3-dimethoxy	–	>64.0	16.0	0.250	synergy
4g	2,4-difluorophenyl	2,3-dimethoxy	–	>64.0	32.0	0.375	synergy
4h	Me	4-Br	–	>64.0	32.0	0.375	synergy
4i	2-furanyl	4-Br	–	>64.0	32.0	0.375	synergy
4j	2,4-difluorophenyl	4-Br	–	>64.0	32.0	0.375	synergy
4k	2-furanyl	4-isopropoxycarbonyl	–	>64.0	32.0	0.375	synergy
4l	2,4-difluorophenyl	4-isopropoxycarbonyl	–	>64.0	32.0	0.375	synergy
4m	H	–	–	>64.0	>64.0	>0.50	indifferent
4n	Me	–	–	>64.0	64.0	>0.50	indifferent
4o	2,4-difluorophenyl	–	–	>64.0	64.0	>0.50	indifferent
19	–	–	–	>64.0	>64.0	>0.50	indifferent
5a	H	2,3-dimethoxy	Me	>64.0	64.0	>0.50	indifferent
5b	H	4-Br	4-bromobenzyl	>64.0	4.0	0.156	synergy
5c	H	4-*t*Bu	Me	>64.0	16.0	0.250	synergy
5d	H	4-Br	2-phenylethyl	>64.0	16.0	0.250	synergy
5e	Ph	4-Br	Me	>64.0	8.0	0.188	synergy
20	–	–	–	>64.0	>64.0	>0.50	indifferent

[a] MIC_{80} value [$\mu g\,mL^{-1}$] of compound in column 1 in combination with 16.0 $\mu g\,mL^{-1}$ fluconazole.

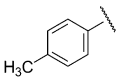
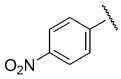
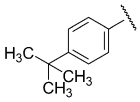
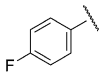
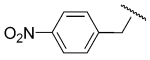
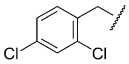
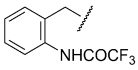
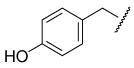
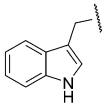
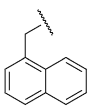
Of compounds **4a**–**o**, compounds **4a**, **4b**, and **4c** exhibited the highest synergistic activities. Analogues **4g**, **4h**, **4i**, **4j**, **4k**, and **4l**, which contained either a 4-bromo or a 4-isopropoxycarbonyl group as the R^2 substituent, showed similar and moderate levels of synergistic activity, whereas compounds **4m**, **4n**, and **4o**, in which the R^2 substituent was a hydrogen atom, showed no activity. This indicated that the opening of the D ring in berberine allowed retention of its synergistic antifungal activity; thus, we were able to deduce that the motif at the C13 position of berberine was not essential to its activity. At this stage in our investigative program, we began to believe that one of the key pharmacophores in berberine resided in its E ring, together with its associated substituents. With this in mind, we proceeded to evaluate the synergistic activities of compound **19** and intermediates **12a**–**d**, which do not have the berberine E ring. As shown in the Supporting Information, compounds **19** and **12a**–**d** did not demonstrate any synergistic activity with fluconazole. Based on these results, we concluded that the E ring in berberine was important to the synergistic activity.

To investigate the effect of the iminium ion in berberine, quaternary ammoniums **3a**–**3e** were prepared from the corresponding inactive tetrahydrogenberberine **10**. These quaternary ammonium compounds **3a**–**e** exhibited synergistic activity. Perhaps more importantly, the nitrogen atom in compounds **3a**–**e** was in the sp^3 hybridization state, and the quaternary ammonium was therefore three-dimensional and bulky, whereas the nitrogen atom in berberine and in compounds **2a**–**j** was in the sp^2 hybridization state, and the quaternary iminium ion was structurally planar. Significant variations in the shape of the quaternary ammonium between compounds **3d** or **3e** and berberine did not lead to much difference in the levels of activity observed. Hence, it was entirely possible that the iminium ion in berberine was not directly involved in the binding of berberine to its target.

Compounds **5a**–**e** were designed and synthesized with a three-dimensional quaternary ammonium in which the nitrogen atom was in the sp^3 hybridization state. Furthermore, the D ring in these compounds was effectively opened by deleting the C13 substituents in compounds **3a**–**e**. As shown in Table 1, compounds **5b**–**e** exhibited synergistic activity, though lower than that of berberine. Compound **20** and intermediates **13a**, **13b**, **14a**, **14b**, **14c**, and **14d**, as shown in the Supporting Information, possessed no synergistic activity with fluconazole. Comparison of compounds **5a**–**e** with compounds **20**, **13a**, **13b**, **14a**–**d**, **3a**–**e** and berberine led us to believe that the E ring of berberine was essential to its activity.

The data collected from preliminary structure–activity relationship (SAR) studies of the analogues outlined above effectively guided us toward the design of compounds **6a**–**e** and **7a**–**i**. Compounds **6a**–**e**, which did not contain an iminium ion, represented berberine analogues with the A, B, and E rings linked together through an amide chain instead of the C and D rings. Unfortunately, however, these compounds possessed no activity in terms of their MIC_{80} and FICI values, as shown in Table 2. Despite this failure, we decided to extend

Table 2. Structures and interaction modes of the title compounds and their MIC_{80} and FICI values.

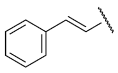
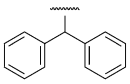
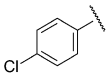
Compd	R^1	R^2	R^3	MIC_{80} [$\mu g\,mL^{-1}$]	MIC_{80} with FLC[a]	FICI	Mode of Interaction
6a	–OCH_2O–			>64.0	>64.0	>0.50	indifferent
6b	–OCH_2O–			>64.0	>64.0	>0.50	indifferent
6c	–OCH_2O–			>64.0	>64.0	>0.50	indifferent
6d	–OCH_2O–			>64.0	>64.0	>0.50	indifferent
6e	–OCH_2O–			>64.0	>64.0	>0.50	indifferent
7a	–OCH_2O–			>64.0	1.0	0.133	synergy
7b	–OCH_2O–			>64.0	0.125	0.126	synergy
7c	–OCH_2O–			>64.0	8	0.188	synergy
7d	–OCH_2O–			>64.0	1.0	0.133	synergy
7e	–OCH_2O–			>64.0	0.5	0.129	synergy
7f	–OCH_2O–			>64.0	1.0	0.133	synergy
7g	–OCH_2O–			>64.0	8.0	0.188	synergy
7h	–OCH_2O–			>64.0	2.0	0.141	synergy
7i	–OCH_2O–			>64.0	16.0	0.250	synergy
7j	–OCH_2O–			>64.0	2.0	0.141	synergy
7k	–OCH_2O–			>64.0	2.0	0.141	synergy
7l	–OCH_2O–			>64.0	1.0	0.133	synergy

our work with the amide linkers by adding different carboxylic acids to give compounds **7a**–**m**. Compounds **7a**–**i** showed excellent levels of activity. Although they did not show antifungal activity ($MIC_{80} > 64.0\ \mu g\,mL^{-1}$) when used alone, their interaction MIC_{80} values decreased to a range of 0.13–16.0 $\mu g\,mL^{-1}$ when they were used in combination with fluconazole at 16.0 $\mu g\,mL^{-1}$. Their FICI values were found to be in the range of 0.126–0.250, indicating that they possessed significant synergistic activity with fluconazole against fluconazole-resistant *C. albicans*. Compounds **7b** and **7e** showed the highest levels of synergistic activity, with interaction MIC_{80} values of 0.125 $\mu g\,mL^{-1}$ and 0.5 $\mu g\,mL^{-1}$ and FICI values of 0.126 and 0.129, respectively. We therefore hypothesized that the A, B, and E rings might be the pharmacophore of berberine, and then proceeded to focus the SAR study on compounds **7a**–**i** by changing or replacing the phenyl ring and the linker.

Replacement of the R^3 substituent with thiophen-2-yl, 1*H*-indol-3-yl, and naphthalen-1-yl led to compounds **7j**, **7k**, and **7l**, respectively, with retention of activity. Replacement of the $CH_2CH_2NHCOCH_2$ motif with $CH_2CH_2NHCONH$ (**8o**, **8p**, and **8q**) or with $CH_2CH_2NHCSNH$ (**8w**) resulted in lower levels of activity than **7a**, **7b**, and **7f**, respectively. Introduction of a hydroxy (**8a**) or phenyl (**8b**) group on the methylene group in the linker of **7a** led to a decrease in activity.

Extended analogues were designed to investigate the effect of the methylenedioxy group in the A ring. For example, the methylenedioxy groups in compounds **7b**, **7d**, **7a**, and **7k** were replaced with dimethoxy groups to give compounds **8c**–**f**, whereas the methylenedioxy

Table 2. (Continued)

Compd	R^1	R^2	R^3	MIC_{80} [μg mL^{-1}]	MIC_{80} with FLC[a]	FICI	Mode of Interaction
7m	–OCH_2O–			>64.0	>64.0	>0.50	indifferent
8a	–OCH_2O–			>64.0	4.0	0.156	synergy
8b	–OCH_2O–			>64.0	2.0	0.141	synergy
8o	–OCH_2O–			>64.0	8.0	0.188	synergy
8p	–OCH_2O–			>64.0	16.0	0.250	synergy
8q	–OCH_2O–			>64.0	16.0	0.250	synergy
8w	–OCH_2O–			>64.0	16.0	0.250	synergy

[a] MIC_{80} value [μg mL^{-1}] of compound in column 1 in combination with 16 μg mL^{-1} fluconazole.

groups in compounds **7b**, **7a**, **8a**, and **7j** were replaced with two hydrogen atoms to give compounds **8g–j**. In one last example, the methylenedioxy groups in compounds **7b**, **7c**, **8a**, and **7j** were replaced by a single hydrogen and a methoxy group to give compounds **8k–n**. All of these analogues showed no activity. Furthermore, in compounds **8r–v** and **8x–z**, replacement of the methylenedioxy group with two hydrogen atoms, or a hydrogen atom and a methoxy group, resulted in no activity at all. Taken together, these results clearly demonstrated that the methylenedioxy group was essential to their activity. The data associated with the inactive compounds **8c–n**, **8r–v**, and **8x–z** is provided in the Supporting Information.

Checkerboard microdilution assay

Some of the compounds displaying good activity in the preliminary screening assay were then subjected to a checkerboard microdilution assay. As a result of this analysis, a series of FICI and interaction MIC_{80} values were determined for the compounds, and the lowest FICI value of each of the tested compounds is shown in Table 3. All compounds tested showed high levels of synergistic activity with fluconazole against fluconazole-resistant *C. albicans*. At concentrations of 1.0, 2.0, 4.0, and 8.0 μg mL^{-1}, all tested compounds provided a significant decrease in the MIC_{80} values of fluconazole from 128.0 μg mL^{-1} to within a range of 0.25–8.0 μg mL^{-1}, as shown in the Supporting Information. Through a comparison of the lowest FICI values of each of the compounds as shown in Table 3, compounds **2g** (0.023), **2j** (0.012), **4c** (0.023), **5b** (0.020), **7a** (0.016), **7b** (0.012), **7d** (0.012), **7e** (0.023), **7j** (0.023), **7f** (0.012), **7l** (0.012), and **8b** (0.012) showed higher levels of synergistic activity than berberine (**1**) (0.035). The lowest FICI value was found for compound **7k** and was similar to that of berberine. It was clear from the data that compounds from series **7** were superior to berberine in terms of their synergistic activity.

Cytotoxicity assay

The synergistically active compounds **1**, **2a**, **2i**, **7b**, **7d**, and **7e** were subjected to a cytotoxicity assay against human umbilical vein endothelial cells (HUVEC) in the presence of fluconazole at a concentration of 16.0 μg mL^{-1}. The results of the assay are reported in Table 4. Berberine (**1**) showed a moderate cytotoxic effect at a concentration of 32.0 μg mL^{-1}, with a viability of 64.0% determined after 24 h and 45.0% after 48 h. Compounds **2a** and **2i** had vi-

Table 3. Checkerboard microdilution assay of fluconazole with the title compounds.

Compd	MIC_{80} [μg mL^{-1}][a]	MIC_{80} with FLC[b]	Lowest FICI	Mode of interaction
1	32.0	1.0(0.5)	0.035	synergy
2g	>64.0	1.0(2.0)	0.023	synergy
2h	32.0	1.0(1.0)	0.039	synergy
2i	32.0	1.0(1.0)	0.039	synergy
2j	>64.0	1.0(0.5)	0.012	synergy
4a	>64.0	1.0(8.0)	0.070	synergy
4b	64.0	1.0(8.0)	0.078	synergy
4c	>64.0	1.0(2.0)	0.023	synergy
5b	>64.0	2.0(0.5)	0.020	synergy
7a	>64.0	1.0(1.0)	0.016	synergy
7b	>64.0	1.0(0.5)	0.012	synergy
7c	>64.0	2.0(32.0)	0.266	synergy
7d	>64.0	1.0(0.5)	0.012	synergy
7e	>64.0	1.0(2.0)	0.023	synergy
7j	>64.0	2.0(1.0)	0.023	synergy
7f	>64.0	1.0(0.5)	0.012	synergy
7h	>64.0	4.0(1.0)	0.039	synergy
7k	>64.0	4.0(0.5)	0.035	synergy
7l	>64.0	1.0(0.5)	0.012	synergy
8b	>64.0	1.0(0.5)	0.012	synergy

[a] Fluconazole-resistant clinical isolates of *C. albicans* 103. [b] Interaction MIC_{80} value of fluconazole given in parentheses.

Table 4. Cytotoxic effects of berberine and its analogues against HUVEC.

Compd	Viability [%][a]			
	32.0 μg mL^{-1}		16.0 μg mL^{-1}	
	24 h	48 h	24 h	48 h
1	64.0 ± 1.7	45.0 ± 1.1	78.0 ± 1.8	52.0 ± 1.5
2a	0	0	0	0
2i	2.0 ± 0.5	0	2.0 ± 0.3	0
7b	90.0 ± 0.1	84.0 ± 1.0	89.0 ± 2.1	86.0 ± 3.0
7d	96.0 ± 1.5	96.0 ± 3.1	96.0 ± 2.5	96.0 ± 2.7
7e	91.0 ± 0.1	91.0 ± 3.0	91.0 ± 0.7	95.0 ± 1.0

[a] Data represent arithmetic means ± SD of at least three independent experiments.

Table 5. Calculated physicochemical properties of compounds **7b**, **7d**, and **7e**.[a]

Property	Optimal Range	**7b**	**7d**	**7e**
M_r [Da]	< 500	328	301	297
log*P*	< 5	2.8	3.0	3.3
H-bond donors	< 5	1	1	1
H-bond acceptors	< 10	7	4	4
Rotatable bonds	< 5	6	5	5
TPSA	< 140	93.4	47.6	47.6

[a] Calculated with Molinspiration property engine ver. 2013.09 (http://www.molinspiration.com).

abilities of 0% and 2.0% after 24 h, and 0% and 0% after 48 h, respectively, showing much higher cytotoxic effects than berberine. In contrast, compounds **7b**, **7d**, and **7e** showed much lower cytotoxic effects with viabilities greater than 84.0% after 24 and 48 h. Berberine has been demonstrated to be more cytotoxic toward a variety of different cell lines than its derivatives containing tertiary amines,[18] indicating that the quaternary iminium ion in berberine is related to its toxicity. Our results are not only consistent with this conclusion, but have also provided compounds with higher levels of activity and lower levels of cytotoxicity by replacing the iminium ion with an amide.

Conclusions

We have described the process of structural optimization of berberine as a synergist to restore antifungal activity of fluconazole against fluconazole-resistant *C. albicans*. Modification of berberine by introducing substituted phenyl groups led to analogues of series **2** and **3**, with synergistic activities. Compound **2a** exhibited higher activity than berberine, but its cytotoxicity (viability: 0%) is much higher than berberine (64%) at a concentration of 32.0 μg mL^{-1}. The SAR study of series **2** and **3** guided us to deconstruct berberine and design series **4** and **5**, with similar or lower activity in general than berberine. The data collected from the preliminary SAR study of the analogues outlined above effectively guided us toward the design of compounds **6a–e** and **7a–i** with novel scaffolds. Series **6** exhibited no activity; however, series **7** was found to possess active synergistic antifungal activity, low levels of cytotoxicity, and good structural characteristics. Subsequent SAR studies of series **7** indicated that the pharmacophore of berberine included the A, B, and E rings, which should be linked using appropriate linkers. Compounds **7b**, **7d**, **7f**, and **7l** (1.0 μg mL^{-1}), in combination with fluconazole, showed remarkable levels of in vitro synergistic antifungal activity against fluconazole-resistant *C. albicans*, providing a decrease in the MIC_{80} values of fluconazole from 128.0 to 0.5 μg mL^{-1}. Compounds **7b**, **7d**, and **7e** demonstrated much lower levels of cytotoxicity toward HUVEC than did parent berberine.

The physicochemical parameters of **7b**, **7d**, and **7e** were calculated using Molinspiration Cheminformatics software (2013), and none were found to violate the optimal requirements for druggability (Table 5). This suggested that these compounds could serve as promising lead compounds for further research.

Experimental Section

Chemistry

All evaporations were conducted in vacuo on a rotary evaporator. Analytical samples were dried in vacuo (1–5 mmHg) at room temperature. Thin-layer chromatography (TLC) was conducted on silica gel 60 F254 plates (Yantai Zhifu Chemical Co. Ltd., Yantai, China). The purities of new compounds were determined using microanalysis (C, H, N) and HPLC and agreed with the theoretical values within ±0.4% and ≥95.0%, respectively. ^{1}H NMR spectra were recorded on a Bruker Avance 300 (300 MHz) spectrometer (Bruker, Fällanden, Switzerland), using $CDCl_3$ or [D_6]DMSO as solvents and tetramethylsilane (TMS) as the internal standard. The purity and electrospray ionization mass spectroscopy (ESIMS) data for the title compounds were determined on an Agilent LC–MS 6120 (Agilent, Santa Clara, CA, USA) coupled to a Max mass spectrometer (Agilent). The following methods were used for liquid chromatography: Method 1 = Ultimate XB-C_{18} column (2.1 mm × 50 mm × 3.5 μm) with a 12.0 min gradient (buffer: TFA (0.01%) + H_2O/CH_3CN), from 90:10 to 70:30 over 0.5 min, and a subsequent gradient from 70:30 to 10:90 over 7.5 min, followed by a gradient from 10:90 to 90:10 over 4.0 min. Method 2 = Ultimate XB-C_{18} column (2.1 mm × 50 mm × 3.5 μm) with a 12 min gradient (buffer: TFA (0.01%) + H_2O/CH_3CN), from 95:5 to 50:50 over 3.0 min, then a subsequent hold at 50:50 for 5.0 min, followed by a gradient from 50:50 to 95:5 over 4.0 min. Compounds **19** and **20** were analyzed using method 2, whereas the other compounds were analyzed using method 1. Compound **15** was purchased from Hengye Zhongyuan Chemical Ltd., (Beijing, China). Compound **17** was purchased from Dengguan Chemical Ltd., (Jintan, China). Compound **18** was purchased from Suzhou Yacoo Chemical Reagent Co. Ltd., (Suzhou, China). The other chemicals were purchased from Aladdin Reagent Co. Ltd., (Shanghai, China) and Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Synthesis of dihydroberberine (9): Berberine (**1**) (3.7 g, 10 mmol) and K_2CO_3 (3.6 g, 30 mmol) were suspended in a mixture of MeOH (60 mL), and a solution of $NaBH_4$ (0.3 g, 7.5 mmol) in 5% aqueous NaOH (5 mL) was added to the mixture in a dropwise manner.[19] The resulting mixture was stirred at room temperature for 1 h. The precipitated product was then filtered, and the filter cake was washed sequentially with a 20% aqueous EtOH mixture (20 mL) and an 80% aqueous EtOH (20 mL) mixture, before being collected and purified by flash chromatography over silica gel, to give title compound **9** (2.6 g, 77%) as a yellow solid.

Synthesis of tetrahydroberberine (10): $NaBH_4$ (1.2 g, 30 mmol) was added in a portionwise manner to a stirred solution of **1** (3.7 g, 10 mmol) and K_2CO_3 (3.6 g, 30 mmol) in MeOH (60 mL), and the resulting mixture was heated at reflux for 1 h, leading to the formation of a yellow solid.[19] The solid was collected by filtration and purified by flash chromatography over silica gel to give title compound **10** (2.5 g, 74%) as a yellow solid.

General procedure for the synthesis of 13-benzylberberine derivatives 2a–j: *o*-Bromo benzyl bromide (1.0 mmol) was added in a dropwise manner to a stirred solution of KI (310 mg, 2.06 mmol) and **9** (337 mg, 1.0 mmol) in CH_3CN (40 mL), and the resulting mixture was held at reflux for 4 h.[19] The reaction mixture was then filtered, and the filtrate was collected and distilled to dryness in vacuo to give the crude residue, which was purified by flash chromatography over neutral alumina to give the final compound **2a** as a yellow solid (350 mg, 60%): ^{1}H NMR (300 MHz, [D_6]DMSO): $\delta =$ 10.08 (s, 1H), 8.13–8.10 (d, 1H, $J = 9.3$ Hz), 7.85–7.83 (d, 1H, $J =$ 9.3 Hz), 7.67–7.64 (d, 1H, $J = 9.3$ Hz), 7.31–7.26 (m, 2H), 7.17 (s, 1H), 6.84–6.81 (m, 1H), 6.71 (s, 1H), 6.08 (s, 2H), 4.88 (s, 2H), 4.61 (2H, s), 4.12 (s, 3H), 4.03 (s, 3H), 3.15 ppm (s, 2H); ESIMS *m/z*: 504.1 $[M-Br]^+$; Anal. ($C_{27}H_{23}Br_2NO_4$) calcd: C 55.41, H 3.96, N 2.39, found: C 55.62, H 3.90, N 2.59. Compounds **2b–j** were also synthesized according to the same general procedure.

General procedure for the synthesis of 7-benyzltetrahydroberberine bromide derivatives 3a–e: A solution of *o*-nitrobenzyl bromide (1.0 mmol) in CH_3CN (40 mL) was added to a stirred solution of **10** (339 mg, 1.0 mmol) in CH_3CN (60 mL) in a dropwise manner, and the resulting reaction mixture was held at reflux for 6 h.[19] The mixture was then cooled and the solvent removed in vacuo to give the crude residue, which was purified by flash chromatography over neutral alumina to give title compound **3a** as a yellow solid (361 mg, 65%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.85–8.83$ (d, 1H, $J = 7.2$ Hz), 7.98–7.95 (d, 1H, $J = 8.1$ Hz), 7.89–7.85 (t, 1H, $J =$ 6.6 Hz), 7.73–7.68 (t, 1H, $J = 6.6$ Hz), 6.91–6.84 (m, 3H), 6.73 (s, 1H), 6.02 (dd, 2H, $J_1 = 1.2$ Hz, $J_2 = 3.6$ Hz), 5.88–5.83 (d, 1H, $J = 13.2$ Hz), 5.74–5.71 (m, 1H), 5.68–5.64 (d, 1H, $J = 13.2$ Hz), 5.19–5.13 (d, 1H, $J = 12.6$ Hz), 4.45–4.40 (d, 1H, $J = 12.6$ Hz), 3.99–3.94 (m, 1H), 3.86 (s, 3H), 3.83 (s, 3H), 3.56–3.40 (m, 2H), 3.37–3.29 (m, 1H), 3.22–3.08 ppm (m, 2H); ESIMS *m/z*: 475.2 $[M-Br]^+$; Purity: 96.7%.

General procedure for the synthesis of 5,6-disubstituted-7,8-dihydro[1,3]dioxolo[4,5-*g*]isoquinolin-6-ium bromide derivatives 4a–o: A solution of **15** (16.5 g, 0.1 mol), HOBt (13.5 g, 0.1 mol), carboxylic acid (0.1 mol), and DCC (20.6 g, 0.1 mol) in THF (200 mL) was stirred at room temperature for 12 h. The mixture was then filtered to remove the white precipitate, and the filtrates were collected and distilled to dryness in vacuo to give the crude residue, which was dissolved in CH_2Cl_2 and washed three times with a saturated solution of Na_2CO_3 before being dried over anhydrous $MgSO_4$ for 1 h. The $MgSO_4$ was then removed by filtration, then evaporated in vacuo to give the crude residue, which was purified by flash chromatography over silica gel to give intermediates **11a–d**, respectively. $POCl_3$ (7.67 g, 50 mmol) was added in a dropwise manner over a period of 0.5 h to a cooled solution (ice bath) of **11a–d** (10 mmol) in toluene (50 mL), and the resulting mixture was stirred at reflux for 12 h. The mixture was then cooled to ambient temperature and quenched by the addition of H_2O (100 mL). The resulting mixture was then adjusted with ammonia to pH 8–9 under stirring, and the resulting aqueous mixture was extracted with CH_2Cl_2 (3×100 mL). The combined organic extracts were then distilled to dryness in vacuo to give the crude residue, which was purified by flash chromatography over silica gel to give intermediates **12a–d**, respectively. Benzyl bromide (2 mmol) was added in a dropwise manner to a solution of corresponding intermediate **12a–d** (2.0 mmol), respectively, in CH_3CN (50 mL), and the resulting mixture was heated at reflux for 6 h.[21] The mixture was then cooled to ambient temperature and the solvent removed in vacuo to give the crude residue, which was purified by flash chromatography over silica gel to give the final compounds **4a–o**, respectively. Compound **4a** was isolated as a yellow solid (543 mg, 58%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.89$ (s, 1H), 7.50–7.49 (m, 1H), 7.43–7.36 (m, 4H), 6.87–6.82 (m, 3H), 6.17–6.15 (d, 2H, $J = 6.9$ Hz), 5.64 (s, 2H), 4.21–4.16 (t, 2H, $J = 7.4$ Hz), 3.50–3.33 (t, 2H, $J = 7.4$ Hz), 1.30 ppm (s, 9H); ESIMS *m/z*: 388.2 $[M-Br]^+$; Purity: 99.6%.

General procedure for the synthesis of 5,6-disubstituted-5,6,7,8-tetrahydro[1,3]dioxolo[4,5-*g*]isoquinolin-6-ium bromide derivatives 5a–e: A solution of 2-benzo[1,3]dioxol-5-ylethylamine **15** (16.5 g, 100 mmol) and the corresponding aldehyde (100 mmol) in formic acid (50 mL) was stirred at reflux for 24 h. The mixture was then cooled to room temperature and diluted with H_2O (100 mL).[22,23] The solution was then adjusted to pH 8–9 with a 1 N aqueous NaOH solution and extracted with CH_2Cl_2 (3×100 mL). The combined organics were then dried for 1 h over anhydrous $MgSO_4$ before being distilled to dryness in vacuo to give the crude residue, which was purified by flash chromatography over silica gel to give intermediate products **13a–b**, respectively, as white solids. Iodomethane or the substituted benzyl bromide was added to a stirred mixture of intermediate **13a** or **13b** (10 mmol) and anhydrous K_2CO_3 (1.38 g, 10 mmol) in EtOH (50 mL), and the resulting mixture was heated at reflux for 12 h. The mixture was then cooled and the resulting precipitation removed by filtration. The filtrates were then collected and distilled to dryness in vacuo to give the crude residue, which was purified by flash chromatography over silica gel to give the intermediate products **14a–d**, respectively, as white solids. 2,3-Dimethoxybenzyl bromide (264 mg, 2.0 mmol) was added in a dropwise manner to a stirred solution of intermediate **14a–d** (2.0 mmol) in CH_3CN (50 mL), and the resulting mixture was heated at reflux for 8 h. The mixture was then cooled and the solvent was removed in vacuo to give the crude residue, which was purified by flash chromatography over silica gel to give products **5a–e**, respectively. Compound **5a** was isolated as a white solid (700 mg, 83%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.44–7.41$ (d, 1H, $J = 8.1$ Hz), 7.13–7.08 (m, 1H), 7.01–6.98 (d, 1H, $J = 8.1$ Hz), 6.59 (s, 1H), 6.50 (s, 1H), 5.91 (s, 2H), 5.17–5.13 (d, 1H, $J = 12.3$ Hz), 5.04–4.00 (d, 1H, $J = 12.3$ Hz), 4.73–4.68 (d, 1H, $J = 15.0$ Hz), 4.50–4.45 (d, 1H, $J = 15.0$ Hz), 4.12–3.92 (m, 2H), 3.87 (s, 3H), 3.84 (s, 3H), 3.19 (s, 3H), 3.15–3.03 ppm (m, 2H); ESIMS *m/z*: 342.1 $[M-Br]^+$; Purity: 95.2%.

General procedure for the synthesis of amide derivatives 6a–e, 7a–m, and 8a–n: A mixture of the starting material **15** (1.65 g, 0.01 mol), HOBt (1.35 g, 0.01 mol), the corresponding carboxylic acid (0.01 mol), and DCC (2.06 g, 0.01 mol) in THF (20 mL) was stirred at room temperature for 12 h. The mixture was then filtered to remove the white precipitate, and the filtrates were collected and distilled to dryness in vacuo to give the crude residue, which was dissolved in CH_2Cl_2 and washed three times with a saturated solution of Na_2CO_3. The organic layers were then dried over anhydrous $MgSO_4$ for 1 h and the solvent removed in vacuo to give the crude residue, which was purified by flash column chromatography over silica gel to give final compounds **6a–d** and **7a–m**, respectively. Compounds **8a–n** were also synthesized according to the same procedure from **16**, **17**, or **18**, respectively. Compound **7a** was isolated as a white solid (2.01 g, 71%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.36–7.25$ (m, 3H), 7.20–7.17 (m, 2H), 6.67–6.64 (d, 1H, $J = 8.1$ Hz), 6.53–6.52 (d, 1H, $J = 1.8$ Hz), 6.45–6.42 (m, 1H), 5.92 (s,

2H), 5.39 (s, 1H), 3.53 (s, 2H), 3.41–3.38 (m, 2H), 2.65–2.60 ppm (t, 2H, J=6.6 Hz); ESIMS m/z: 284.1 $(M+H)^+$; Purity: 96.8%.

General procedure for the synthesis of urea derivatives 8o–v: The corresponding isocyanate (0.01 mmol) was added to a cooled solution (ice bath) of starting material **15, 16**, **17**, or **18** (0.01 mol) in THF (50 mL), respectively, and the resulting mixture was stirred at room temperature for 6–12 h.[25] The solvent was then removed in vacuo to give the crude residue, which was purified by recrystallization to give final products **8o–v**, respectively. Compound **8o** was isolated as a white solid (2.61 g, 82%): ^{1}H NMR (300 MHz, $CDCl_3$): δ=8.02–7.99 (d, 1H, J=8.1 Hz), 7.36–7.33 (d, 1H, J=1.5 Hz), 7.24–7.21 (m, 1H), 7.02–6.97 (m, 1H), 6.77–6.71 (m, 1H), 6.67–6.64(m, 2H), 5.94 (s, 2H), 3.52–3.48 (t, 2H, J=6.6 Hz), 2.82–2.77 ppm (t, 2H, J=6.6 Hz); ESIMS m/z: 319.1 $[M+H]^+$; Purity: 99.4%.

General procedure for the synthesis of thiourea derivatives 8w–z: The corresponding isothiocyanate (0.01 mmol) was added to a cooled (ice bath) solution of starting material **15**, **16**, **17**, or **18** (0.01 mol) in THF (50 mL), respectively, and the resulting mixture was stirred at room temperature for 6–12 h.[25] The solvent was then removed in vacuo to give the crude residue, which was purified by recrystallization to give final products **8w–z**, respectively. Compound **8w** was isolated as a white solid (1.38 g, 46%): ^{1}H NMR (300 MHz, $CDCl_3$): δ=8.29 (s, 1H), 7.34–7.31 (t, 2H, J=3.9 Hz), 7.24–7.22 (t, 1H, J=3.9 Hz), 7.05–7.04 (d, 2H, J=3.9 Hz), 6.67–6.65 (d, 2H, J=3.9 Hz), 6.61 (s, 1H), 6.54–6.53 (d, 1H, J=3.9 Hz), 6.01 (s, 1H), 5.89 (s, 2H), 3.82–3.78 (q, 2H, J=3.0 Hz), 2.80–2.78 ppm (t, 2H, J=3.0 Hz); ESIMS m/z: 301.1 $(M+H)^+$; Purity: 99.7%.

Synthesis of compounds 19 and 20: CH_3I (0.6 mL, 5.0 mmol) was added to a solution of intermediate **12a** or **14a** (2.0 mmol) in CH_3CN (50 mL), respectively, and the resulting mixture was heated at reflux for 8 h.[19] The mixture was then cooled, and the precipitate was filtered and washed sequentially with CH_2Cl_2 and EtOAc to give desired product **19** or **20**, respectively. Compound **19** was isolated as a yellow solid (285 mg, 90%): ^{1}H NMR (300 MHz, D_2O): δ=8.59 (s, 1H), 7.12 (s, 1H), 6.90 (s, 1H), 6.08 (s, 2H), 3.92–3.86 (t, 2H, J=8.1 Hz), 3.63 (s, 3H). 3.15–3.10 ppm (t, 2H, J=8.1 Hz); ESIMS m/z: 190.1 $[M-I]^+$; Purity: 97.2%. Compound **20** was isolated as a light-yellow solid (546 mg, 82%): ^{1}H NMR (300 MHz, D_2O): δ=6.75 (s, 1H,), 6.62 (s, 1H), 5.91 (s, 2H), 4.40 (s, 2H), 3.62–3.57 (t, 2H, J=6.6 Hz), 3.13–3.06 ppm (m, 8H); ESIMS m/z: 206.1 $[M-I]^+$; Purity: 95.3%.

Biological activity assays

Strains and agents stock solution preparation: Four clinical isolates of fluconazole resistant *C. albicans* (MIC_{80}=128.0 μg mL^{-1}) were used in this study, and *C. albicans* ATCC 90028 was used as a quality control. The strains were cultured at 30 °C under constant shaking (200 rpm) in a liquid complete medium (YPD) consisting of 1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone, and 2% (*w*/*v*) dextrose. The stock solution of fluconazole (Pfizer, Dalian, China) was prepared in sterile water, whereas the other compounds, including berberine (Sigma–Aldrich, MO, USA), were prepared in DMSO.

Antifungal susceptibility testing: The in vitro MIC_{80} values of the compounds against the clinical isolates of *C. albicans* were determined using the microbroth dilution method, according to the Clinical and Laboratory Standards Institute.[12,26] The initial concentration of the fungal suspension in RPMI1640 medium was 10^3 colony-forming units (CFU)/mL, and final compound concentrations were in the range of 1.0–8.0 μg mL^{-1}. The final concentration of fluconazole was set at 16.0 μg mL^{-1} for the preliminary screening assay, and in the range of 0.125–64.0 μg mL^{-1} for the checkerboard microdilution assay. The assay plates were incubated at 35 °C for 24 h. Optical density was measured at 630 nm, and the background optical densities were then subtracted from the value provided for each well. Each isolate was tested in triplicate. The MIC_{80} values were determined as the lowest concentrations of the drugs (alone or in combination) that inhibited fungal growth by 80% compared with that of the drug-free wells.

Cytotoxicity assays: Human umbilical vein endothelial cells (HUVEC) were maintained in RPMI1640 medium (HyClone, Beijing, China) supplemented with 10% fetal calf serum (Gibco, CA, USA), and 100 μg mL^{-1} streptomycin. The cells were grown in a humidified incubator in 5% CO_2 at 37 °C and were used for assays during their exponential growth phase. Cell viability was determined following 24 h and 48 h of incubation with berberine (16.0 or 32.0 μg mL^{-1}) or its derivatives in the presence of fluconazole (16.0 μg mL^{-1}) using a Roche Cell Proliferation Kit II (XTT). Briefly, the XTT mixing solution (XTT labeling reagent/electron coupling reagent, 5:1) was added to each well containing 5×10^3 HUVEC. After a 6 h reaction, optical density was measured by a microplate reader (Thermo, MA, USA) at 450 nm. The cytotoxic activity was measured by the following formula: viability (%) = 100 × OD_{450} of test/OD_{450} of control.

Acknowledgements

This research was supported by the National Natural Science Fund of China (Nos. 21072226, 30672533, 30772644, and 21272270) and the Shanghai Pujiang Program (No. 05PJ14003).

Keywords: antifungal agents · berberine · *Candida albicans* · drug resistance · structure–activity relationships

[1] O. Gudlaugsson, S. Gillespie, K. Lee, J. V. Berg, J. F. Hu, S. Messer, L. Herwaldt, M. Pfaller, D. Diekema, *Clin. Infect. Dis.* **2003**, *37*, 1172–1177.
[2] S. K. Fridkin, W. R. Jarvis, *Clin. Microbiol. Rev.* **1996**, *9*, 499–511.
[3] C. Charlier, E. Hart, A. Lefort, P. Ribaud, F. Dromer, D. W. Denning, O. Lortholary, *J. Antimicrob. Chemother.* **2006**, *57*, 384–410.
[4] A. Goffeau, *Nature* **2008**, *452*, 541–542.
[5] B. C. Monk, A. Goffeau, *Science* **2008**, *321*, 367–369.
[6] M. Nucci, J. R. Perfect, *Clin. Infect. Dis.* **2008**, *46*, 1426–1433.
[7] W. Youngsaye, B. Vincent, C. L. Hartland, B. J. Morgan, S. J. Bahrlage, S. Johnston, J. A. Bittker, L. Macpherson, S. Dandapani, M. Palmer, L. Whitesell, S. Lindquist, S. L. Schreiber, B. Munoz, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 5502–5505.
[8] S. Gamarra, E. M. F. Rocha, Y. Q. Zhang, S. Park, R. Rao, D. S. Perlin, *Antimicrob. Agents Chemother.* **2010**, *54*, 1753–1761.
[9] P. Uppuluri, J. Nett, J. Heitman, D. Andes, *Antimicrob. Agents Chemother.* **2008**, *52*, 1127–1132.
[10] J. Jin, N. Guo, J. Zhang, Y. Ding, X. Tang, J. Liang, L. Li, X. Deng, L. Yu, *Lett. Appl. Microbiol.* **2010**, *51*, 351–357.
[11] N. Guo, X. Wu, L. Yu, J. Liu, R. Meng, J. Jin, H. Lu, X. Wang, S. Yan, X. Deng, *FEMS Immunol. Med. Microbiol.* **2010**, *58*, 193–201.
[12] H. Quan, Y. Y. Cao, Z. Xu, J. X. Zhao, P. H. Gao, X. F. Qin, Y. Y. Jiang, *Antimicrob. Agents Chemother.* **2006**, *50*, 1096–1099.
[13] Y. Han, J. H. Lee, *Biol. Pharm. Bull.* **2005**, *28*, 541–544.
[14] S. K. Battu, M. A. Repka, S. Maddineni, A. G. Chittiboyina, M. A. Avery, S. Majumdar, *AAPS PharmSciTech* **2010**, *11*, 1466–1475.
[15] X. Zhang, F. Qiu, J. Jiang, C. Gao, Y. Tan, *Xenobiotica* **2011**, *41*, 290–296.
[16] W. Hua, L. Ding, Y. Chen, B. Gong, J. He, G. Xu, *J. Pharm. Biomed. Anal.* **2007**, *44*, 931–937.
[17] M. M. Kheir, Y. Wang, L. Hua, J. Hu, L. Li, L. Du, *Food Chem. Toxicol.* **2010**, *48*, 1105–1110.

[18] L. Orfila, M. Rodrı'guez, T. Colman, M. Hasegawa, E. Merentes, F. Arvelo, *J. Ethnopharmacol.* **2000**, *71*, 449 – 456.
[19] Y. H. Li, P. Yang, W. J. Kong, Y. X. Wang, C. Q. Hu, Z. Y. Zuo, Y. M. Wang, H. Gao, L. M. Gao, Y. C. Feng, N. N. Du, Y. Liu, D. Q. Song, J. D. Jiang, *J. Med. Chem.* **2009**, *52*, 492 – 501.
[20] A. S. Capilla, M. Romero, M. D. Pujol, D. H. Caignard, P. Renard, *Tetrahedron* **2001**, *57*, 8297 – 8303.
[21] J. Tóth, A. Dancsó, G. Blaskó, L. Tőke, P. W. Groundwater, M. Nyerges, *Tetrahedron* **2006**, *62*, 5725 – 5735.
[22] F. I. Zubkov, J. D. Ershova, A. A. Orlova, V. P. Zaytsev, E. V. Nikitina, A. S. Peregudov, A. V. Gurbanov, R. S. Borisov, V. N. Khrustalev, A. M. Maharramov, A. V. A. Varlamov, *Tetrahedron* **2009**, *65*, 3789 – 3803.
[23] A. Yokoyama, T. Ohwada, K. Shudo, *J. Org. Chem.* **1999**, *64*, 611 – 617.
[24] X. Zhao, M. X. Jia, X. K. Jiang, L. Z. Wu, Z. T. Li, G. J. Chen, *J. Org. Chem.* **2004**, *69*, 270 – 279.
[25] J. M. Vega-Pérez, I. Periñán, M. Argandoña, M. Vega-Holm, C. Palo-Nieto, E. Burgos-Morón, M. López-Lázaro, C. Vargas, J. J. Nieto, F. Iglesias-Guerra, *Eur. J. Med. Chem.* **2012**, *58*, 591 – 612.
[26] P. A. Wayne, *Approved standard M27-A3*: Clinical and Laboratory Standards Institute (CLSI): USA, **2008**.
[27] F. C. Odds, *J. Antimicrob. Chemother.* **2003**, *52*, 1 – 1.

Received: August 9, 2013
Revised: October 10, 2013
Published online on November 4, 2013