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Discovery of diazahexa/hepta cyclic cage-like compounds with broad-spectrum antifungal activity against *Candida* and *Cryptococcus* species†

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Invasive fungal infections caused by *Candida* and *Cryptococcus* species lead to life threating infections in immunocompromised individuals. Furthermore, increasing incidence of fungal strains resistant to FDA-approved antifungal drugs along with the paucity of antifungal drugs warrants novel drugs to treat invasive fungal infections. In this study, we investigated the antifungal activity of a novel series of diazahexa/hepta cyclic cage-like compounds. Results indicate that compounds with unsubstituted and *o*-methyl substitution on aryl rings exhibit potent broad-spectrum antifungal activity against various fungal strains. In addition, these compounds showed significant inhibitory activity against *Candida* hyphae and biofilm formation. Collectively, results from this study indicate that these compounds are promising candidates to develop as novel antifungal drugs to treat drug-resistant fungal infections.

Introduction

Fungal infections, especially invasive candidiasis, are among the most common blood stream infections in the hospital setting, particularly among cancer patients and patients in intensive care units.[1–4] *Cryptococcus* spp. also demonstrates high prevalence in immunocompromised patients infected with human immunodeficiency virus (HIV).[5] With increased dependence and utilization of broad-spectrum antifungal drugs, there has been a causal increase in resistance to antifungal drugs across *Candida* and *Cryptococcus* species.[6–8] However, with the most recent antifungal drug approved in the 2000s, only a limited number of antifungal drugs are currently available to treat these fungal infections.[9,10] Coupled with the fungal strains developing resistance to current drugs and paucity of FDA-approved antifungal agents, an unmet and urgent need to develop new antifungal drugs are necessary to treat the systemic invasive fungal infections.[10–12]

In this study, we aim to identify novel compounds against the important human fungal pathogens, *Candida* and *Cryptococcus* species. Our results indicate that compounds **5a** and **5g** exhibit potent inhibitory activity against fungal strains and have the potential to develop as new antifungal drugs.

Results and discussion

Chemistry

Recently, we synthesized a series of diazahexa-/hepta cyclic cage-like compounds (Fig. 1) *via* domino multicomponent protocol[13] that involved (i) 1,3-dipolar cycloaddition and (ii) concomitant annulation steps (Scheme 1). 1,3-Dipolar cycloaddition reaction of the azomethine ylide generated *in situ* from an equimolar amount of acenapthenequinone (**1**) and L-phenylalanine (**2**), with a series of bisbenzylidenepiperidin-4-ones **3** in methanol under reflux condition afforded the cage-

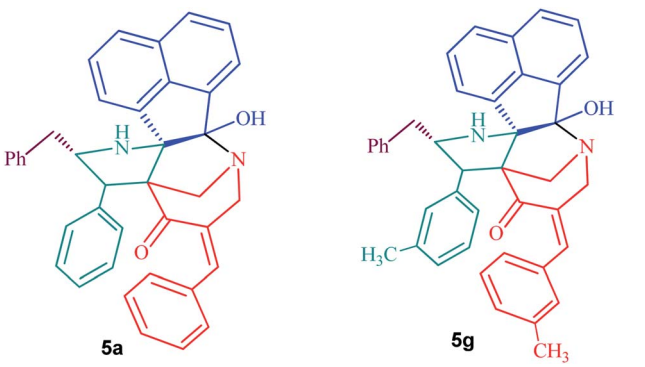

Fig. 1 Structure of potent diazahexacyclic cage compounds **5a** and **5g**.

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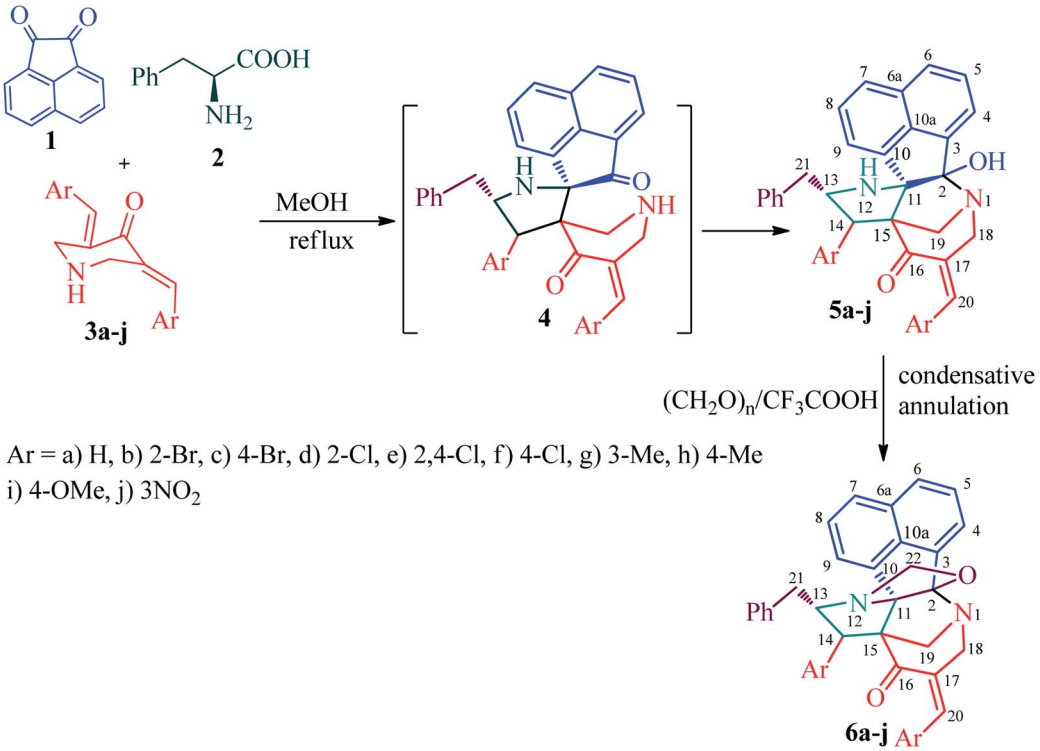


Scheme 1 Synthesis of diazahexa-/hepta cyclic cage compounds, **5a–j** and **6a–j**.

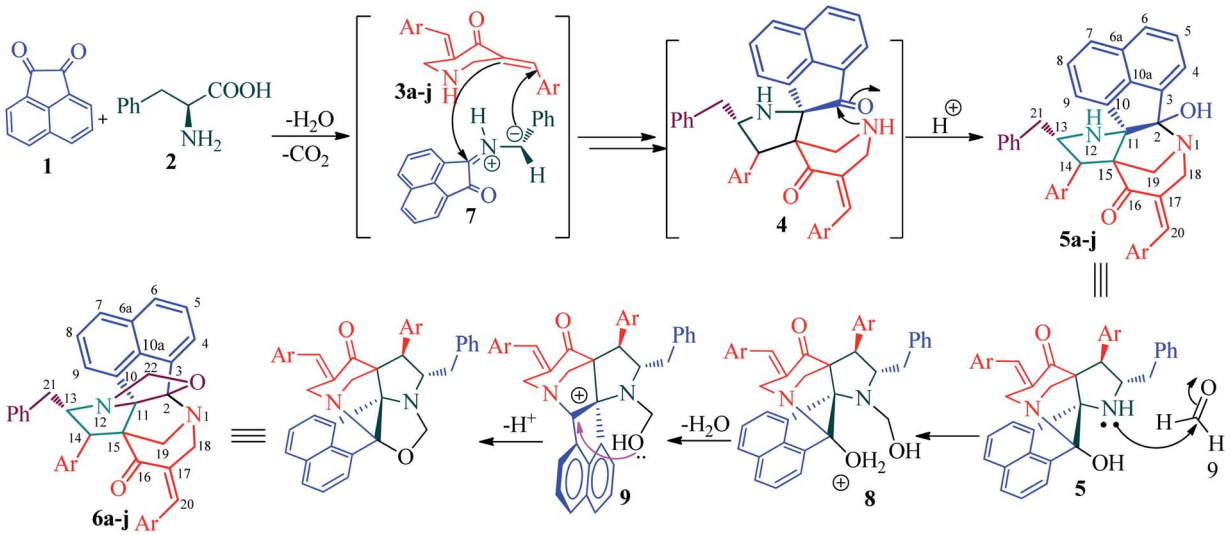
like systems in good to excellent yields. The crude cage-like product **5** obtained was purified through column chromatography and its structure was confirmed by spectroscopic studies. With a series of diaza hexacyclic cage-like compound **5** in hand, we proceeded further to explore their reaction with paraformaldehyde in the presence of trifluoroacetic acid, which led to the formation of structurally interesting diazahepta cyclic cage-like compounds **6** comprising [1,2-*c*]oxazolidine, pyrrolidine, and piperidine structural units. In a typical reaction, diazahexacyclic cage-like compounds **5** (1 mmol) were treated overnight with paraformaldehyde (1 mmol) in the presence of a catalytic amount trifluoroacetic acid (10 mol%) in CH_2Cl_2 at room temperature which afforded **6** in excellent yields (88–98%). The structure of the synthesized compound was confirmed by ^1H and ^{13}C NMR spectroscopic studies and their stereochemistry has been unambiguously ascertained by single X-ray crystal analysis (Fig. 2).[14] The plausible mechanism for the formation of diazahexa/hepta cyclic cage-like compounds **5** and **6** is described in Scheme 2.

Biology

Determining the antifungal activity of novel compounds. Preliminary screening of diazahexa/hepta cyclic cage system compounds synthesized in our laboratory was tested for its antifungal activity by mean inhibitory concentration (MIC) assay as described before.[15] MIC for all the strains against indicated compounds were done in triplicates. Compounds **5** and **6** series were screened against the *C. albicans* ATCC 10231 strain. Results indicate that compounds **5a** and **5g** demonstrated antifungal activity with an MIC of 4 μg mL^{-1} (Table 1).

Next, we tested the antifungal activity of compounds **5a** and **5g** against clinical strains of *Candida* spp. and *Cryptococcus neoformans* in order to determine if the compounds possess broad-spectrum activity against a variety of fungal species. After 24 hours of incubation, both **5a** and **5g** demonstrated potent antifungal activity against *C. albicans*, *Candida parapsilosis*, and *Candida tropicalis* with the MICs ranging from 0.5 to 4 μg mL^{-1} (Table 2). Further, **5a** and **5g** also inhibited the growth of these fungal strains even after 48 hours of incubation with the MICs ranging from 2 to 8 μg mL^{-1} (Table 2). Compounds **5a** and **5g** also showed excellent antifungal activity against *C. neoformans* and *C. glabrata* with the MICs ranging from 0.5 to 2 μg mL^{-1} after 48 hours of incubation (Table 2).

Fluconazole was used as a metric of comparison, due to its status as FDA-approved antifungal drug with widespread clinical use. Fluconazole inhibited the *Candida* strains with a wide range of MICs from 0.0625 to 16 μg mL^{-1} after 24 hours of incubation (Table 2). However, after 48 hours, the MICs of fluconazole for all strains were increased several fold with most strains inhibited at 32 μg mL^{-1} (Table 2). Surprisingly, unlike fluconazole, both compounds **5a** and **5g** showed potent inhibitory activity against most of the *Candida* strains, even after 48 hours of incubation without considerable increase in the MIC values. In addition, **5a** and **5g** also showed excellent antifungal activity against *C. glabrata* and *C. neoformans* after 48 hours of incubation compared to fluconazole (Table 2). Collectively, compounds **5a** and **5g** exhibit potent broad-spectrum antifungal activity compared to fluconazole against all the fungal strains tested in this study.



Scheme 2 Plausible mechanism for the formation of diazahexa-/heptacyclic cage system.

Compounds 5a and 5g inhibit *C. albicans* hyphae formation and attachment. The morphological switching ability of *C. albicans* from yeast to hyphae form is a key factor in the virulence of these pathogenic fungi.[16–18] Therefore, we tested the effect of compounds against *C. albicans* hyphae formation. *C. albicans* ATCC 10231 strain was grown in hyphae inducing medium containing fetal bovine serum (FBS) in the presence or absence of compounds and the hyphae formation and attachment was determined using the crystal violet assay as described before.[19,20] Results indicate that all compounds significantly inhibited the hyphae formation and attachment (Fig. 3). At a concentration of 128 μg mL^{-1}, compounds **5a**, **5g** and fluconazole significantly inhibited the hyphae formation and attachment by 90, 50 and 70% respectively (Fig. 3). At 256 μg mL^{-1}, compounds **5a** and **5g** showed almost complete inhibition (more than 90%) of hyphae formation (Fig. 3). However, increasing the concentration of fluconazole (256 μg mL^{-1}) did not improve the inhibitory activity (Fig. 3). Taken together, compounds **5a** and **5g** showed potent inhibitory effect on *C. albicans* hyphae formation and attachment in a concentration dependent manner, and showed excellent activity compared to fluconazole.

Table 1 Screening compounds for antifungal activity against *C. albicans* ATCC 10231

Entry	Compounds	MIC (μg mL^{-1})
1	**5a**	4
2	**5b**	128
3	**5c**	32
4	**5d**	128
5	**5e**	128
6	**5f**	128
7	**5g**	4
8	**5h**	256
9	**5i**	16
10	**5j**	16
11	**6a**	16
12	**6c**	64
14	**6d**	128
15	**6g**	64
16	**6i**	16

Compounds 5a and 5g inhibit the metabolic activity of fungal cells in *C. albicans biofilm*. Increased resistance by *Candida* spp. to antifungal therapies has been partially attributed to biofilm formation.[21,22] Fungal biofilms can also lead to recurrent infections.[23,24] Therefore, we investigated the effect of compounds on *C. albicans* biofilm formation using an MTS reduction assay. Results indicate that **5a**, **5g** and fluconazole significantly inhibited the metabolic activity of fungal cells in *C. albicans* biofilm (Fig. 4). Compounds **5a** and **5g** at the concentrations of 128 and 256 μg mL^{-1} inhibited the metabolic activity of fungal cells in the biofilm by 40 and 30% respectively (Fig. 4). Fluconazole also showed significant inhibition (30%) at a concentration of 256 μg mL^{-1} (Fig. 4).

Cytotoxicity activity of compounds against mammalian cells. Fungi are eukaryotic pathogens, therefore developing therapeutic agents capable of inhibiting pathogenic fungal growth without mammalian host toxicity a significant challenge. Therefore, we determined to examine the cytotoxicity activity of compounds against mammalian cell lines using a MTS assay. Surprisingly, our results indicate that compounds **5a** and **5g** were not toxic to all the tested mammalian cell lines including human colon cancer cell line (HCT 116), mouse mammary gland cell line (4T1), mouse fibroblast cell line (CT26) and mouse lung cell line (LLC1) cells up to 256 μg mL^{-1} (Fig. 5). Thus, it appears evident that compounds **5a** and **5g** possess antifungal activity without causing toxicity to mammalian cells.

Results from this study indicate that compounds **5a** and **5g** possess potent antifungal and antivirulence activities without causing mammalian host toxicity. In addition, **5a** and **5g** showed excellent activity compared to fluconazole. Further studies to determine the pharmacokinetic and physicochemical

Table 2 MICs of compounds and fluconazole against clinical isolates of *Candida* spp. and *C. neoformans*[a]

Strain	Description	MIC Fluconazole (μg mL^{-1})		MIC **5a** (μg mL^{-1})		MIC **5g** (μg mL^{-1})	
		24 h	48 h	24 h	48 h	24 h	48 h
C. albicans NR-29434	Bloodstream isolate from a patient with candidemia from Winnipeg, Manitoba, Canada in 2000	0.125	32	4	8	2	4
C. albicans NR-29449	Vaginal isolate from a patient with vaginitis from Ann Arbor, Michigan, USA between 1990 and 1992	0.5	32	4	8	4	8
C. albicans NR-29435	Bloodstream isolate from a patient with candidemia from Iowa City, Iowa, USA in 2000	0.5	0.5	2	4	2	4
C. albicans NR-29448	Clinical isolate from a person with candidemia from Arizona, USA	1	32	2	8	2	4
C. albicans NR-29437	Bloodstream isolate from a patient with candidemia from Brussels, Belgium in 2000	0.25	32	4	8	2	4
C. albicans NR-29446	Bloodstream isolate from a patient with candidemia from Utah, USA	16	32	1	4	0.5	4
C. albicans NR5-29453	Clinical isolate from a patient with thrush and HIV from Pretoria, South Africa	0.0625	0.0625	4	4	2	4
C. albicans NR-29438	Bloodstream isolate from a patient with candidemia from Tel-Hashomer, Israel, in 2000	0.0625	0.25	4	8	2	4
C. albicans NR-29367	Clinical isolate from China	0.0625	0.0625	4	4	2	4
C. albicans NR-29439	Bloodstream isolate from a patient with candidemia from Omaha, Nebraska, USA, in 2000	0.5	32	2	8	2	4
C. albicans NR-29440	Bloodstream isolate from a patient with candidemia from Lille, France, in 2000	0.25	0.5	4	8	2	4
C. albicans NR-29441	Bloodstream isolate from a patient with candidemia from Iowa City, Iowa, USA, in 2000	0.25	16	2	8	1	4
C. albicans NR-29442	Bloodstream isolate from a patient with candidemia from Ottawa, Ontario, Canada, in 2000	0.25	32	4	8	2	4
C. albicans NR-29444	Oral isolate from a patient with vaginitis collected in Ann Arbor, Michigan, USA between 1990 and 1992.	1	16	2	4	2	4
C. parapsilosis ATCC 22019	Clinical isolate from a patient with celiac disease from Puerto Rico	1	4	0.5	2	0.5	2
C. tropicalis ATCC 13803	FDA provided isolate	8	32	4	8	2	4
C. glabrata ATCC 90030	Bloodstream isolate from a patient from Iowa	n.d.	4	n.d.	0.5	n.d.	0.5
C. albicans ATCC 10231	Clinical isolate from a patient with bronchomycosis	2	4	2	4	1	4
C. neoformans NR-41291	Cerebrospinal fluid isolate from a patient from China in July 2011	n.d.	32	n.d.	2	n.d.	2
C. neoformans NR-41292	Cerebrospinal fluid isolate from a patient from China in February 2012	n.d.	32	n.d.	1	n.d.	1
C. neoformans NR-41296	Cerebrospinal fluid isolate from a patient from China in February 2012	n.d.	4	n.d.	1	n.d.	1
C. neoformans NR-41295	Cerebrospinal fluid isolate from a patient from China in February 2012	n.d.	32	n.d.	1	n.d.	1
C. neoformans NR-41294	Cerebrospinal fluid isolate from a patient from China in June 2011	n.d.	4	n.d.	0.5	n.d.	0.5
C. neoformans NR-41297	Cerebrospinal fluid isolate from a patient from China in February 2012	n.d.	8	n.d.	1	n.d.	1
C. neoformans NR-41298	Cerebrospinal fluid isolate from a patient from China in February 2012	n.d.	16	n.d.	2	n.d.	2
C. neoformans NR-41299	Cerebrospinal fluid isolate from a patient from China in August 2009	n.d.	8	n.d.	1	n.d.	1

[a] Not detected (n.d.).

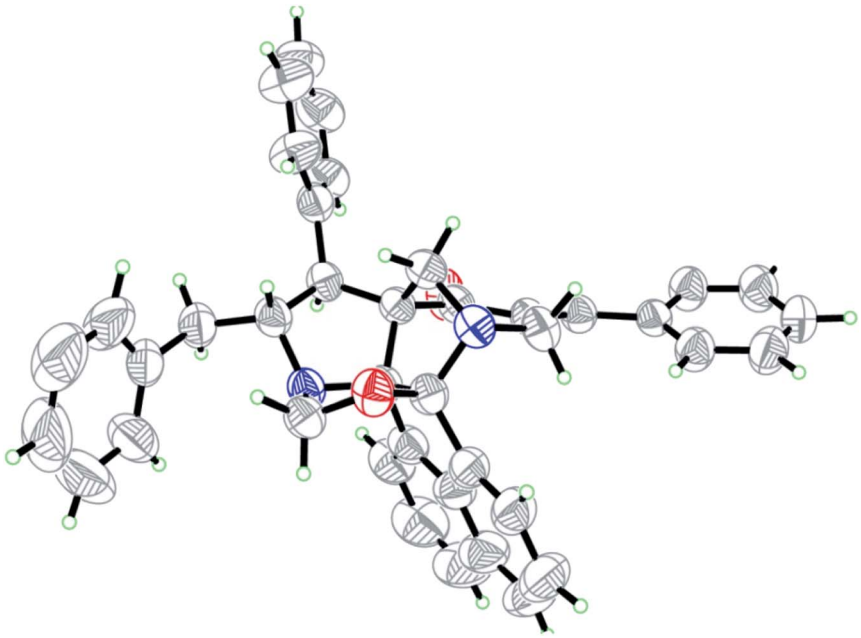


Fig. 2 ORTEP diagram of compound **6a**.

profile of these compounds is essential to move these compounds to the next stage of the drug development pipeline. The structure of **5a** and **5g** contain the nitrogen (N1) atom in the diazahexa cage-like compounds, a characteristic shared by the benzylamine and allylamine antifungals. These antifungals act as inhibitors of squalene epoxidase, a key enzyme in the synthesis of sterols by fungi. The nitrogen (N12) atom in the pyrrolidine ring shares similar bonding to that of echinocandins, which act as beta-1, 3-D-glucan synthase inhibitors. The carbonyl unit of piperidone at position sixteen shares similarities to that of the flavonoids, which have become a new area of study for their antifungal activity. Therefore, we speculate that these novel compounds may target pathways specific to fungi including fungal 3b-glucan synthase and ergosterol biosynthesis.[25] However, future studies will be needed to understand the antifungal mechanism of these compounds. In addition, further structure modifications to enhance the activities of **5a** and **5g** should be also a promising avenue. Taken

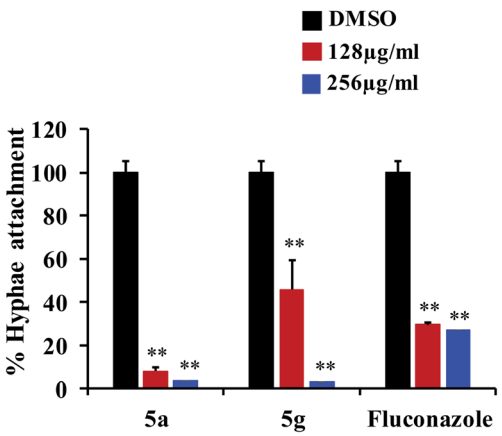


Fig. 3 Activity of compounds **5a** and **5g** against *C. albicans* hyphae formation and attachment. *C. albicans* ATCC 10231 was incubated with the indicated compounds or DMSO for 12 hours in the hyphae inducing conditions and the adherent hyphae was stained using crystal violet. Absorbance measured at 490 nm and the percent hyphae formation and attachment in treatment groups was determined relative to DMSO treated control groups. Experiments were repeated atleast three times in triplicates and the data represented as means ± SEM of all replicates. The statistical significance with *P* values (* ≤ 0.05, ** ≤ 0.01) were considered significant as per *t*-test.

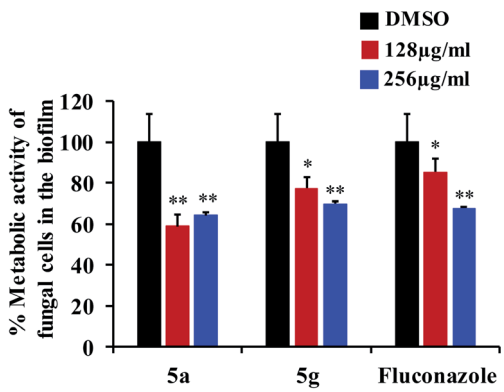


Fig. 4 Effect of compounds **5a** and **5g** on the metabolic activity of *C. albicans* cells in fungal biofilm. *C. albicans* ATCC 10231 was incubated under biofilm inducing conditions in the presence or absence of indicated compounds or DMSO and after 48 hours of incubation the metabolic activity of fungal cells in the biofilm was determined using the MTS assay. Percent metabolic activity in the treatment groups was calculated in relative to the DMSO control group. Experiments were repeated atleast three times in triplicates and the data represented as means ± SEM of all replicates. The statistical significance with *P* values (* ≤ 0.05, ** ≤ 0.01) were considered significant as per *t*-test.

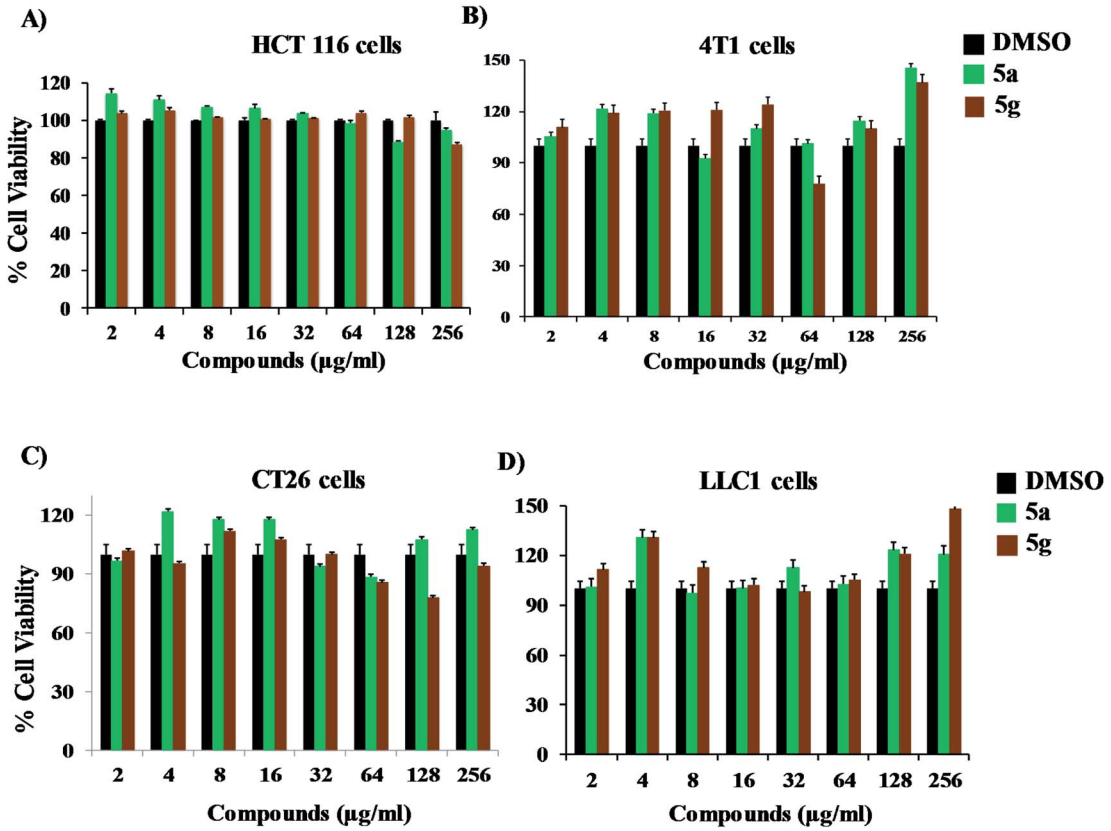


Fig. 5 Cytotoxicity assay in mammalian cells. Mammalian cell lines including human colon cancer cell line (HCT 116), mouse mammary gland cell line (4T1), mouse fibroblast cell line (CT26) and mouse lung cell line (LLC1) cells (A, B, C and D respectively) were incubated with the indicated concentrations of compounds and, after 24 hours of incubation, the cell viability was measured using the MTS assay. Percent cell viability was calculated relative to the DMSO treated control groups. Experiments were repeated atleast three times in triplicates and the data represented as means ± SEM of all replicates. The statistical significance with P values (* ≤ 0.05, ** ≤ 0.01) were considered significant as per t-test.

together, compounds **5a** and **5g** have strong potential to develop as novel antifungal drugs.

Materials and methods

Chemistry

General procedure for the synthesis of hexacyclic compounds 5a–j. An equimolar mixture of 3,5-bisarylmethylidene pyridinone (1 mmol), acenaphthenequinone (1 mmol) and L-phenylalanine (1 mmol) was dissolved in MeOH (15 mL) and refluxed for 2 h. After completion of the reaction evident by TLC, diazahexacyclic cage compound were afforded in pure form through column chromatography using EtOAc : hexane (7 : 3 v/v) as eluent.

General procedure for the synthesis of heptacyclic compounds 6a–g. An equimolar mixture of hexacyclic compound **5** (1 mmol) and paraformaldehyde (1 mmol) was dissolved in CH_2Cl_2 (5 mL) and then added a catalytic amount of trifluoroacetic acid (10 mol%). The mixture was stirred overnight at room temperature. After completion of the reaction as evident from TLC, diazahexacyclic cage compound were afforded in pure form through column chromatography using EtOAc : hexane (1 : 4 v/v) as the eluent and recrystallized from EtOAc.

Biology

MIC assay. Mean inhibitory concentration (MIC) was performed as per CLSI standards as described before.[15,26,27] Briefly, fungal strains were suspended in phosphate buffered saline to match a 0.5 McFarland standard (about 10^6 CFU mL^{-1} approximately), and then diluted in RPMI 1640 to create a 10^2 to 10^3 CFU mL^{-1} solution. 100 µL of this inoculated solution was placed in the wells of a 96-well untreated plate and incubated for 24 and 48 hours in the presence or absence of indicated compounds listed in Table 2 or DMSO. MIC was recorded after 24 and 48 hours of incubation.

Hyphae assay. The effect of compounds on *Candida* hyphae formation and attachment was performed as described previously.[19,20] Briefly, *C. albicans* ATCC 10231 strain was incubated for 16 hours at 37 °C in hyphae inducing media (30% fetal bovine serum, 70% RPMI 1640) in a 96-well untreated plate in the presence or absence of indicated compounds or DMSO control. After incubation, wells were washed and stained with 0.02% crystal violet and the absorbance measured at 490 nm using a spectrophotometer. Percent hyphae attachment was calculated relative to the vehicle control groups.

Biofilm assay. The effect of compounds on *C. albicans* biofilm formation was determined using *C. albicans* ATCC 10231 as

descried elsewhere.[19,20,27,28] *C. albicans* was resuspended in RPMI 1640 medium at a concentration of 3×10^6 cells per mL, then 100 μL of inoculum were transferred to the wells of a 96-well treated plate and incubated in the presence or absence of indicated compounds. The plate was incubated for 48 hours at 37 °C and the metabolic activity of fungal cells in *C. albicans* biofilm was determined using MTS assay. Absorbance was measured at 490 nm using a spectrophotometer and the percent metabolic activity was determined in relative to the DMSO treated control groups.

Cytotoxicity assay. The toxic effect of compounds against mammalian cells was investigated using human colon cancer cell line (HCT 116), mouse mammary gland cell line (4T1), mouse fibroblast cell line (CT26) and mouse lung cell line (LLC1) cells as described previously.[15,27] HCT116 and LLC1 cells were cultured in FBS-supplemented DMEM media, 4T1 and CT26 cells were cultured in FBS-supplemented RPMI media containing penicillin–streptomycin at 37 °C for 24 hours in the presence or absence of indicated concentrations of compounds. Cell viability was measured by MTS assay and the percent viable cells in the treatment group was determined relative to the DMSO control groups.

Statistical analysis. Statistical significance was assessed using student's *t*-test and *P* values (* $\leq$ 0.05, ** $\leq$ 0.01) were considered as significant.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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