Synthesis of Novel Benzimidazolium Gemini Surfactants and Evaluation of Their Anti-Candida Activity

7 authors, including:

Farooq Wani
Jamia Millia Islamia
13 PUBLICATIONS   55 CITATIONS

SEE PROFILE

Amad Uddin
Jamia Millia Islamia
10 PUBLICATIONS   23 CITATIONS

SEE PROFILE

Babita Aneja
Jamia Millia Islamia
22 PUBLICATIONS   139 CITATIONS

SEE PROFILE

Gerard Sheehan
University of Birmingham
28 PUBLICATIONS   185 CITATIONS

SEE PROFILE

Some of the authors of this publication are also working on these related projects:

Project Medicinal Chemistry View project

Project Molecular docking View project

Synthesis of Novel Benzimidazolium Gemini Surfactants and Evaluation of Their *Anti-Candida* Activity

Farooq Ahmad Wani,[†,‖] Amaduddin,[‡] Babita Aneja,[‡] Gerard Sheehan,[§] Kevin Kavanagh,[§] Rabia Ahmad,[‖] Mohammad Abid,*[,‡] and Rajan Patel*[,†]

[†]Biophysical Chemistry Laboratory, Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia (A Central University), New Delhi 110025, India

[‡]Medicinal Chemistry Laboratory, Department of Biosciences, and [‖]Department of Chemistry, Jamia Millia Islamia, New Delhi 110025, India

[§]Department of Biology, Maynooth University, Co Kildare 045, Ireland

S *Supporting Information*

ABSTRACT: Owing to the rise in antimicrobial and chemotherapeutic drug resistance, there is a desperate need to formulate newer as well as more effective agents. With this perspective, here we outline the synthesis of two novel gemini surfactants with different substitutions at the nitrogen atom of the benzimidazolium ring. Both the compounds induced significant reductions in *Candida* growth in various yeast strains. The reduction in *Candida* growth seemed likely through the reduction in ergosterol biosynthesis: a sterol constituent of yeast cell membranes. Different concentrations of both compounds were used to determine the cellular ergosterol content which indicates an important disordering of the ergosterol biosynthetic pathway. Cytotoxic studies were carried out using HEK 293 (human embryonic-kidney cells) and *Galleria mellonella* larvae (an in vivo model of antimicrobial studies). Administration of both the compounds to *G. mellonella* larvae diseased by the yeast *Candida albicans* resulted in increased survival indicating their in vivo activity.
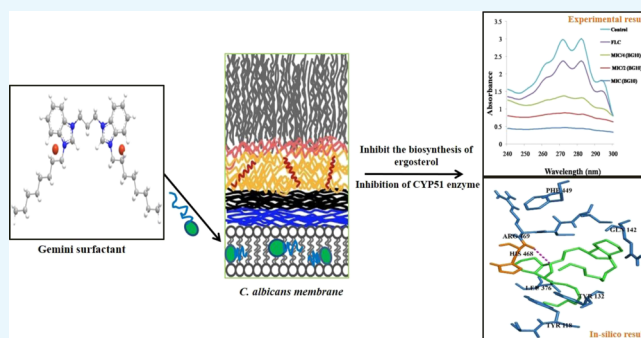
1. INTRODUCTION

One of the serious problems people currently face in daily life is their inexorable exposure to several bacteria, fungi, and many other micro-organisms. The emergence of resistant micro-organisms against available antimicrobial agents is a matter of serious concern.[1] Persistent fungal infections are connected with higher death/mortality rates in immuno-compromised hosts, such as cancer patients, organ transplantation, and acquired immuno deficiency syndrome.[2] The range of effective and safer antifungal agents is limited, despite the high death rate associated with fungal infections.[3] Conventional antifungal agents, that is, fluconazole (FLC), caspofungin, and amphotericin B have some therapeutic limits like related drug toxicity, uneven bioavailability, and absence of oral/intravenous preparations. More significantly, increasing resistance to the majority of the clinically used antifungal agents has considerably minimized the outcome rate of antifungal therapy leading to higher mortality.[4] Thus, the identification of harmless and effective antifungal agents to gear up challenges of increasing frequency/incidences of fungal infections is immediately required.[4,5] Gemini surfactants are novel surfactants which have drawn much attention from both academic and industrial research communities and possess effective antimicrobial activity.[6,7] These are amphiphilic in nature having two hydrophilic moieties as well as two hydrophobic tails joined by a spacer who may be stiff or stretchy.[8−10] Gemini surfactants have improved physicochemical features like lesser critical micelle concentration (cmc), high efficiency of adsorption, better wetting and foaming ability, solubilizing power, as well as soap lime-dispersing properties,[11−15] wetting agent, emulsifier, fabric softener, and foaming agents.[16−19] Gemini surfactants are more efficient in reducing surface tension and form micelles comparatively at lower concentrations as compared to monomeric surfactants.[20,21]

The surfactants with the quaternary ammonium head group have been extensively used as antimicrobial agents over the decades.[22,23] These surfactants kill the microorganisms mostly because of electrostatic and hydrophobic interactions on the cell surfaces[24] by intercalating the cell membrane and changing its molecular organization, which increases membrane permeability and thus results in cytoplasmic diffusion and

cell lysis.[25] The cytotoxicity and green behavior of the gemini surfactants are of immense importance ahead of their use in any potential field.[26−28] Although various reports are centered on their antimicrobial activities,[29−31] the exact means of action is not yet completely understood. Consequently, the main aim of this study was to design and synthesize new gemini surfactants and evaluate their antifungal activity. Therefore, in this work, we have synthesized two novel benzimidazolium-based gemini surfactants 1,3-bis(1,1-dialkyl benzimidazolium)-propane bromide expressed as BG8 and BG10, respectively, and characterized by Fourier transform infrared (FT-IR), NMR, and mass spectroscopy (Scheme S1). The purity of these surfactants was checked by high performance liquid chromatography. The physicochemical characterization was also performed using tensiometry given in the Supporting Information. The *anti-candida* studies for these compounds against three strains of *Candida* (*Candida albicans*, *Candida glabrata*, and *Candida tropicalis*) along with one fluconazole (FLC) resistant *C. albicans* strain were performed. The following Scheme 1 shows the probable mechanism of action of gemini benzimidazolium surfactants with yeast membrane.

Scheme 1. Probable Mechanism of Action of Gemini Benzimidazolium Surfactants with a Yeast Membrane

Ergosterol

Gemini surfactant

Gemini-Ergosterol complex

Membrane Disruption by Gemini surfactant
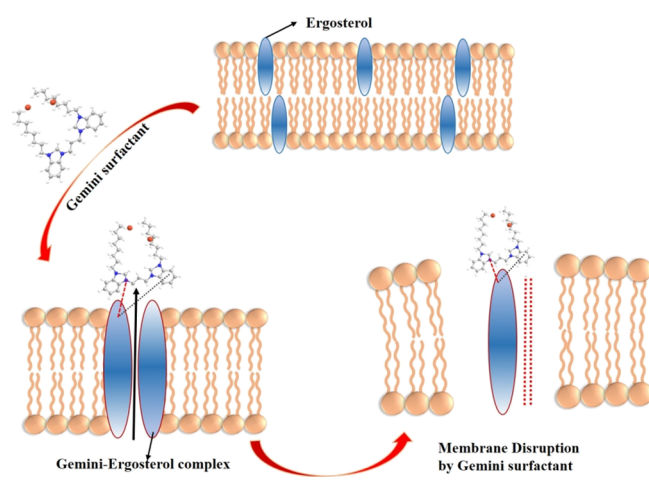
2. RESULTS AND DISCUSSION

2.1. Anti-candida Studies. *2.1.1. In Vitro Anti-candida Activity.* The *anti-candida* studies for these compounds against three strains of *Candida* (*C. albicans*, *C. glabrata*, and *C. tropicalis*) along with one FLC resistant *C. albicans* strain were performed and the results suggested that gemini benzimidazolium surfactants showed better *anti-candida* potential as compared to the reference drug FLC. Both surfactants, BG8 and BG10 also exhibited potent inhibitory activity against the resistant strain with minimum inhibitory concentration (MIC) value of 23.7 and 10.9 μM, respectively, indicating that BG10 is more effective against the resistant *C. albicans* strain compared with standard drug FLC (Table 1). BG8 exhibited a MIC value of 23.7 μM against all the three sensitive strains. Moreover, BG10 was observed to be more effective than that of BG8 and reference drug FLC 51 μM against *C. tropicalis* strains with a value of MIC 10.9 μM, respectively. Because it is renowned that *C. albicans* is to blame for 50−60% cases of the pathological condition of fungus infection (candidiasis),[30,32] we concentrate our studies on the *C. albicans* strain. Based on

Table 1. MIC Values (in μM) of BG8 and BG10 against Different Strains of *Candida*[a]

| strains	BG8	BG10	FLC
C. albicans ATCC90028	23.7	21.8	51.0
C. glabrata ATCC90030	23.7	10.9	25.5
C. tropicalis ATCC750	23.7	10.9	51.0
FLC-resistant *C. albicans*	23.7	10.9	3265

[a]*C. albicans* resistant = clinical isolates of *C. albicans* (FLC resistant); FLC = fluconazole.

the results obtained with standard *Candida* strains, these surfactants were further investigated against FLC-resistant *C. albicans*. Similar results were also reported by Ruiz et al.[32] They reported that green cationic gemini surfactants N^{α},N^{ω}-bis(N^{α},caproylarginine)α,ω-propyldiamide (C3(CA)2) and lichenysin exhibited antimicrobial synergies against *C. albicans*, *Bacillus subtilis*, *Yersinia enterocolitica*, and *Escherichia coli*, and observed that C3(CA)2-lichenysin exhibited lesser MIC values than the individual ones. Zhang et al. has also reported the antibacterial as well as antifungal activities of gemini surfactant monomers and copolymers with better MICs than the conventional surfactants.[33]

2.1.2. Growth Curve. To investigate the impact of those potent surfactants (BG8 and BG10) on the expansion of *Candida* cells, growth curve studies were performed. Completely different concentrations of the test compounds (2MIC, MIC, and MIC/2) were exposed on the *Candida* cells (Figure 1a,b). The positive and negative controls were taken as FLC-treated cells and untreated cells, respectively. The *Candida* cells do not show any noteworthy growth once exposed to MIC and 2MIC concentrations of these surfactants with the incessant lag stage of 24 h. But, at sub-MIC concentrations of BG8 and BG10, growth has been found after 7 and 12 h, respectively, for a standard *C. albicans* strain. In the case of the FLC-resistant *Candida* strain, growth has been observed after 10 and 14 h when exposed to sub-MIC concentrations of BG8 and BG10, respectively. Moreover, FLC exhibited less efficacy in inhibiting the growth of FLC-resistant *Candida* cells than BG8 and BG10. The above results reveal that BG10 was observed to be a good inhibitor against *Candida* cell growth as compared to BG8 as shown in Figure 1c,d.

2.1.3. Synergistic Study. The in vitro synergistic activity of *anti-candida* in grouping with FLC was also performed against standard and FLC-resistant *C. albicans* strains. The outcomes revealed that *anti-candida* activity of each surfactant BG8 and BG10 against the standard *C. albicans* strain was significantly improved when used in a mixture with FLC. The fractional inhibitory concentration index (FICI) values of surfactants BG8 and BG10 were 0.253 and 0.127 against the standard *C. albicans* strain indicating moderate synergy of BG8 and a highly synergistic effect of BG10 (Table 2). Moreover, BG10 also exhibited potential synergistic activity with a FICI value of 0.250 against the FLC-resistant *C. albicans* strain while BG8 also shows synergy in combination with FLC in the same strain (Tables 2 and 3).[34,35]

2.1.4. Ergosterol Biosynthesis in C. albicans. The effect of these compounds on ergosterol biosynthesis in *C. albicans* was examined in order to determine whether they affected cell membrane biosynthesis. The quantitative total sterol profiling in *C. albicans* ATCC 90028 was determined as described by an earlier stated method.[36] FLC was taken as the positive control. The results clearly demonstrated a decrease in dose-dependent
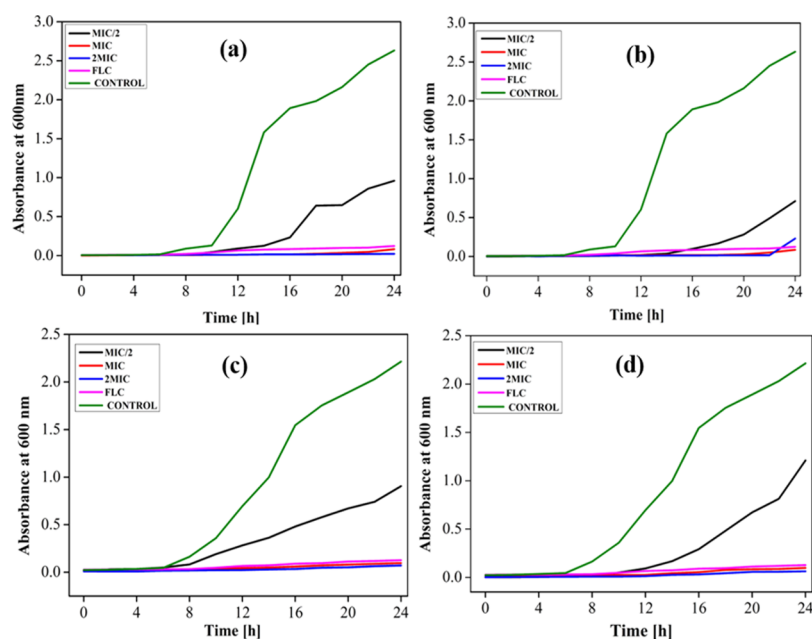
Figure 1. Dose-dependent growth curve of *C. albicans* ATCC-90028 in the presence of (a) BG8 and (b) BG10, FLC-resistant *C. albicans* D15.9 in the presence of (c) BG8 and (d) BG10.

Table 2. In Vitro Synergistic *Anti-candida* Activity of BG8

compound	MIC alone (μM)		MIC in combination (μM)		FICI[a]	mode of interaction
	BG8	FLC	BG8	FLC		
C. albicans (ATCC 90028)	23.7	51.0	5.29	12.7	0.253	synergy
C. albicans D15.9[a]	23.7	3265	11.86	816	0.250	synergy

[a]FICI = fractional inhibitory concentration index; synergy and antagonism were defined by FICI ≤ 0.5 and >4, respectively. Indifference was defined by 0.5 < FICI ≤ 4.

Table 3. In Vitro Synergistic *Anti-candida* Activity of BG10

| compound	MIC alone (μM)		MIC in combination (μM)		FICI[a]	mode of interaction
	BG10	FLC	BG10	FLC		
C. albicans (ATCC 90028)	21.8	51.0	2.73	6.36	0.127	synergy
C. albicans D15.9[a]	10.9	3265	1.36	816	0.250	synergy

[a]FICI = fractional inhibitory concentration index; synergy and antagonism were defined by FICI ≤ 0.5 and >4, respectively. Indifference was defined by 0.5 < FICI ≤ 4.

ergosterol content as cells were grown in variable concentrations of BG compounds. It can be inferred from the results that the exposure of cells to BG compounds with MIC and MIC/2 levels expressively inhibited ergosterol biosynthesis (Figure 2a,b). However, these compounds are comparatively less effective at low concentrations (MIC/4). Moreover, BG8 and BG10 inhibited the total ergosterol synthesis content more effectively than FLC at all the tested concentrations.

2.2. In Vivo Studies. *2.2.1. Toxicity of BG8 and BG10 Gemini Surfactants in Galleria mellonella Larvae.* Further, to evaluate the in vivo toxicity of these compounds, larvae of *Galleria mellonella* were inoculated with 20 μL of BG8 and BG10, and viability was assessed over 96 h. At concentrations ranging from 3030 to 70 μM, these compounds were nontoxic in the *G. mellonella* larvae, yielding 100 ± 0% viability after 96 h (Figure 3a,c).

2.2.2. Antifungal Efficacy of BG Compounds in the G. mellonella Larvae. To estimate the in vivo *anti-candida* efficiency of BG compounds, the *G. mellonella* larvae was infected by an inoculum of *C. albicans* (5×10^5 per 20 μL). One hour post infection, the larvae were administered with 20 μL of BG compounds (3030−70 μM) or 20 μL of dimethyl sulphoxide (DMSO) (10% v/v) control, and viability was assessed over 96 h. BG8 at 76 μM increased the viability of the *G. mellonella* larvae to 76.66 ± 3.33% at 48 h and 53.00 ± 3.33% compared to the respective DMSO-injected controls (48 h; 66.66 ± 3.33%, 72 h; 33.33 ± 6.66%). BG8 at concentrations ranging from 70 to 3030 μM significantly increased the viability of the *G. mellonella* larvae following with *C. albicans* infection. Concentrations of BG10 of 70−3030 μM significantly increased larval viability at 48 h (100 ± 0%, $p >$ 0.01). A concentration of 151 μM increased larval viability at 72 h (76.66 ± 3.33%, $p > 0.05$), as compared to the relative DMSO control (33.33 ± 6.66%). BG10 was effective in blocking *C. albicans*-induced larval mortality at 48 h (100% ± 0%, $p > 0.01$) Figure 3b,d. The above outcomes indicate that administration of BG8 and BG10 to the larvae produced no adverse effects (e.g., melanisation) or death which is a good indication that they may be nontoxic in mammals. Administration of compounds to larvae infected with *C. albicans* resulted in increased survival, indicating in vivo activity. This has been reported previously with novel antimicrobial compounds[37,38] and a strong relationship among the in vivo activity of compounds in larvae and rats has previously been demonstrated.[39]
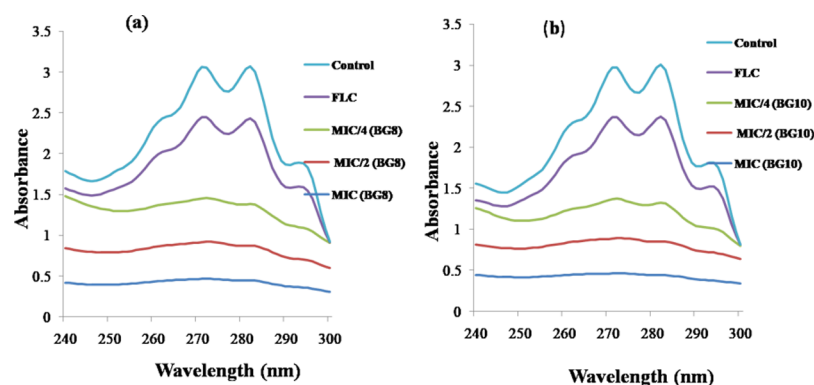
Figure 2. UV spectrophotometric sterol profiles of *C. albicans* ATCC-90028 after treatment with various concentrations (MIC/4, MIC/2, and MIC) of test compounds (a) BG8 and (b) BG10.
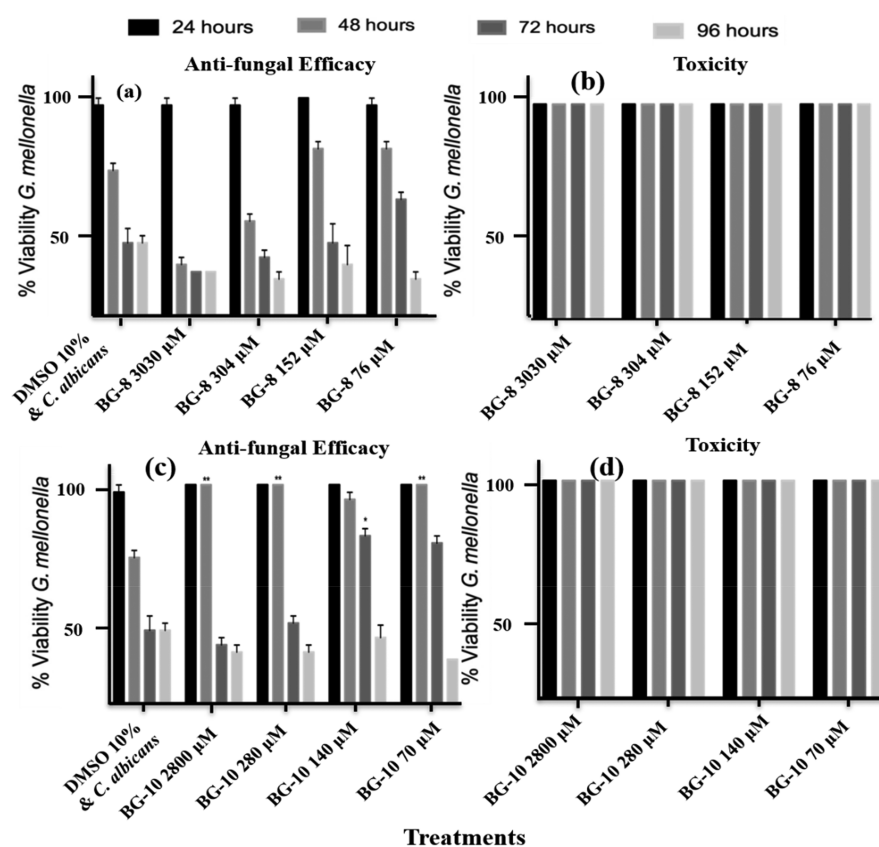
Figure 3. Toxicity of BG8 (a) and BG10 (b) gemini surfactants in *G. Mellonella* larvae and antifungal efficacy of BG8 (c) and BG10 (d) gemini surfactants in *G. Mellonella* larvae.

2.3. In Silico Studies. The effect of these compounds on ergosterol biosynthesis was further confirmed by in silico studies in which both gemini surfactants were docked with an enzyme lanosterol 14-α demethylase (CYP51) member of the cytochrome P-450 super family that catalyzes the oxidative elimination of the 14-α alkyl group from lanosterol in the ergosterol biosynthesis pathway. The inhibition of CYP51 directly depleted ergosterol followed by the inhibition of yeast growth and acts as a primary target of azoles as antifungal drugs. The CYP51 crystal structure from pathogenic yeast *C. albicans* was recently published by the group of Keniya et al. and submitted to RCSB protein data bank (PDB ID: 5v5z).[40] The interaction and binding energy of inhibitors BG8 and BG10 with CYP51 were identified using molecular docking

(Figure 4), and the docked results were compared with reference drug itraconazole. Both gemini surfactants contain the benzimidazolium group flanked by long alkyl hydrophobic side chains (C_8H_{17} in BG8 and $C_{10}H_{21}$ in BG10). The docking studies revealed that BG8 was bound close to the heme group in the active site of CYP51 interacting with active site residues such as Phe-449, Ile-471, Gln-142, Tyr-132, Leu-376, and forms a hydrogen bond with His-468 with a binding affinity of -7.7 kcal mol^{-1}. While as BG10 was bound to residues Tyr-447, Phe-105, Glu-115, Arg-469, His-310, and a hydrogen bond with GLY-303 with a binding affinity of -8.3 kcal mol^{-1} as compared to itraconazole shows -9.6 kcal mol^{-1}.[41] Thus, the docking results support BG8, BG10 binding with the 14-α demethylase enzyme (CYP51) and inhibiting ergosterol
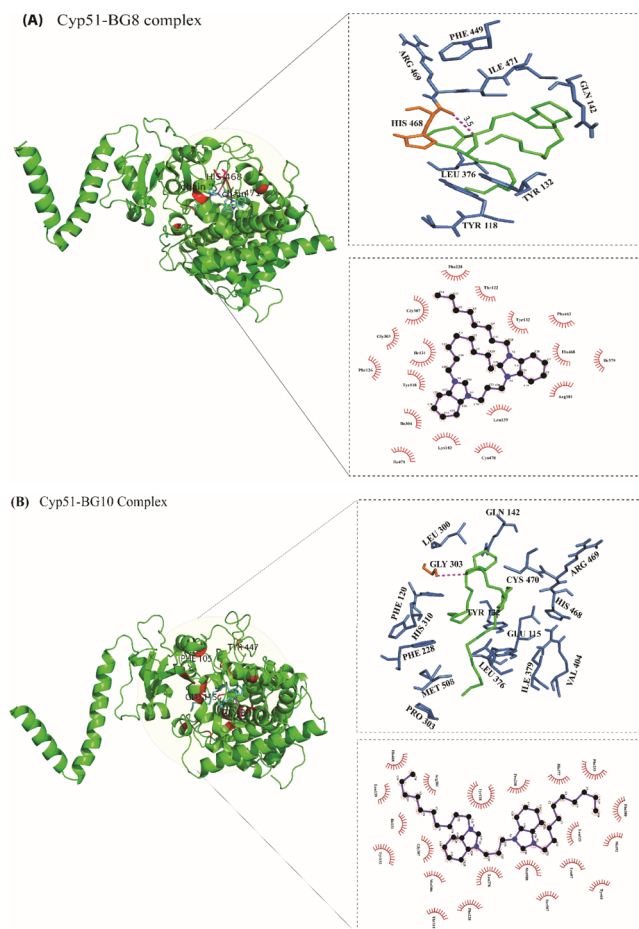
Figure 4. Cartoon model, 2D and ligplot of (A) CYP51−BG8 and (B) CYP51−BG10 complexes.

biosynthesis. The probable mechanism of action for gemini benzimidazolium surfactants with the yeast membrane is shown in Scheme 1.

2.4. Toxicity. *2.4.1. Hemolytic Assay.* The toxicity of surfactants BG8 and BG10 on human red blood cells (hRBCs) were observed to evaluate the hemolytic assay. At 1510 μM concentration, BG8 and BG10 showed reasonable toxicity triggering only 28 and 33% hemolysis, respectively, as compared to the reference drug FLC with 78% hemolysis. However, at 152 μM concentration, these surfactants show low toxicity triggering only 13% (BG8) and 16% (BG10) cell lysis. At 38 μM concentration, BG8 and BG10 are totally harmless causing only 4 and 8% cell lysis, respectively. Thus, these potent *anti-candida* geminis exhibited less toxicity than the reference drug FLC at each tested concentration extending from 38 to 1510 μM showing an extra protection index, as compared to reference drug FLC (Figure 5a).

2.4.2. Cytotoxicity Assay. The selected compounds were further evaluated for cytotoxicity by the (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay on the noncancerous human cell line (HEK293), which is a specific cell line initially resulting from the cells of the human embryonic kidney grown by a tissue culture. HEK 293 cells are widely used in cell biological research over decades, because of their consistent growth and inclination for transfection.[42−44] The selected compounds were screened in the concentration range of 0−160 μM, and it was found that even at 160 μM, compounds BG8 and BG10 do not disrupt the feasibility of
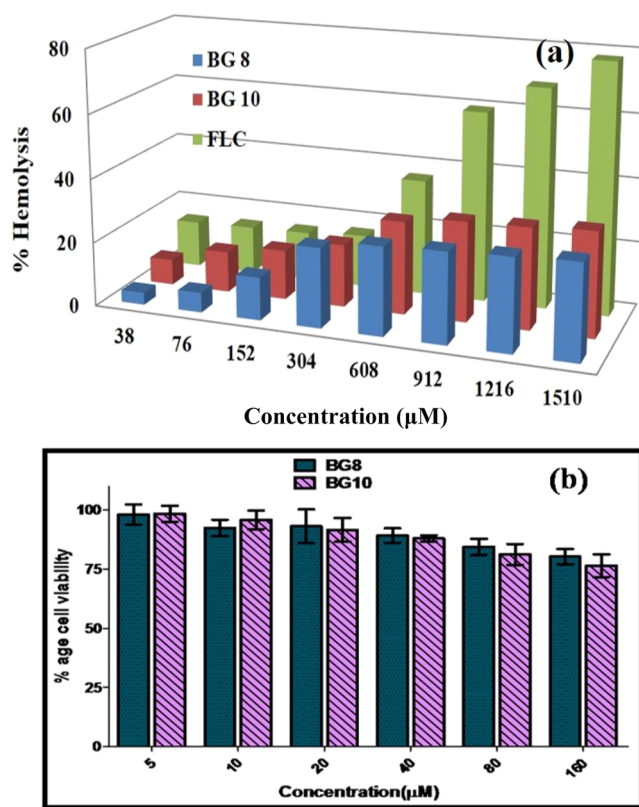
Figure 5. Hemolytic assays (a) and cell viability assay (b) of BG8 and BG10.

HEK293 in a significant manner. These results clearly advocated that the selected compounds are noncytotoxic to HEK293 cells in the tested concentration range. Further, these results speculate that these compounds might be used as potential antifungal molecules, as in the tested sub-micromolar concentration range, these exhibited a noncytotoxic nature towards normal human cells (Figure 5b).[30]

3. CONCLUSIONS

Two benzimidazolium-based gemini surfactants BG8 and BG10 have been synthesized and were well characterized by FT-IR, ^{1}H NMR, ^{13}C NMR, and mass spectroscopy. Moreover, their physicochemical characterization was also done using a surface tension method to evaluate various interfacial parameters viz: Γ_{max}, pC_{20}, A_{min}, and P (packing parameter). The *anti-candida* studies for these compounds along with reference drug, FLC against three strains of *Candida* (*C. albicans*, *C. glabrata,* and *C. tropicalis*) were performed and the results suggested that gemini BG surfactants showed better *anti-candida* potential as compared to the reference drug. BG8 and BG10 exhibit potent inhibitory activity against the resistant strain with MIC values of 23.7 and 10.9 μM, respectively, indicating that BG10 is more effective against the resistant *C. albicans* strain compared with standard drug FLC. Administration of these compounds to larvae infected with *C. albicans* resulted in increased survival indicating in vivo activity. The consequence of these compounds on ergosterol biosynthesis in *C. albicans* was examined to determine affected cell membrane biosynthesis and the results confirmed a decrease in dose-dependent ergosterol content once the cells had been grown in variable concentrations of test compounds. Our docking studies further confirmed the ergosterol biosynthesis

Table 4. Surface Active Parameters of BG8 and BG10

surfactants	cmc (mM)	γ_{cmc} (mN m^{-1})	π_{cmc} (mN m^{-1})	pC_{20}	Γ_{max} (10^3 mol m^{-2})	A_{min} (nm^2)	P
BG8	13	35.75	39.6	1.57	2.22	0.74	0.45
BG10	1.5	32.4	40.4	3.35	1.58	1.05	0.32

in *C. albicans*, revealing that both BG8 and BG10 were bound close to the heme group in the active site of CYP51 with high binding affinities. The MTT assay with noncancerous human cell line (HEK293) results indicated that the selected compounds are noncytotoxic to the HEK293 cells in the tested concentration range.

4. EXPERIMENTAL SECTION

4.1. Materials. Benzimidazole (purity 99% Sigma-Aldrich USA), 1-bromooctane, and 1-bromodecane (purity 99% Sigma-Aldrich USA). The reagents were used without any extra purification. Milli-Q H$_2$O with specific conductivity <1.4 μS cm^{-1} was used throughout the experiments.

4.2. Synthesis. 1,3-Bis(1-octylbenzimidazolium)propane bromide (C$_{34}$H$_{53}$Br$_2$N$_4$) simply represented as BG8 and 1,3-bis(1-decylbenzimidazolium)propane bromide (C$_{38}$H$_{63}$Br$_2$N$_4$) represented as BG10 were synthesized by the procedure given in the Supporting Information. The FT-IR, NMR and mass spectra were used to confirm the structures of all the products.

The spectral results of FT-IR, ^{1}H NMR, ^{13}CNMR, and mass are given below. (Figures S1−S6)

4.2.1. BG8 Gemini 1,3-Bis(1-octylbenzimidazolium)-propane Bromide (C$_{34}$H$_{53}$Br$_2$N$_4$). FT-IR (4000−600 cm^{-1} ATR tablet) 3050 cm^{-1} [v(Ar-H)], 2915 cm^{-1} [v(CH$_3$)], 2848 cm^{-1} [v(CH$_2$)], 2031 cm^{-1} [$v(\pi)$] that is exocyclic C═C vibration, 1410 cm^{-1} [v(C═N)], 1349 cm^{-1} [v(C═C bending)], 1192 cm^{-1} [v(C−C bending)], 888 cm^{-1} [v(C−H bending)] and at 3324 cm^{-1} is due to the absorbed water.

^{1}H NMR (CDCl$_3$-δ = 7.5 ppm) δ = 0.75 ppm (t, 6H), 1.25 (s, 4H), 1.6 (m, 16H), 3.6 (q, 4H), 4.5 (t, 4H), 5.8 (d, 4H), 7.8 (m, 4H), 9.2 (d, 4H), 10.2 (s, 2H), 10.9(t, 4H).

^{13}C NMR (CDCl$_3$-δ = 80 ppm) δ = 13.94 ppm (CH$_3$), 22.4 (CH$_2$), 28.90 (CH$_2$), 30.10 (CH$_2$), 32.0 (CH$_2$), 50 (CH$_2$), 115.9 (CH), 127.2 (C attached at the bridged position), 131.2 (CH of the six-membered ring), 139.9 (CH$_2$), 140 (CH═).

ESI-MS (LCMS): C$_{33}$H$_{50}$N$_4$Br$_2$ found 649, calcd 660; C$_{26}$H$_{35}$N$_4$Br$_2$ 539, calcd 557; C$_7$H$_{15}$ 112, calcd 99.

4.2.2. BG10 Gemini 1,3-Bis(1-decylbenzimidazolium)-propane Bromide (C$_{38}$H$_{63}$Br$_2$N$_4$). FT-IR (4000−600 cm^{-1} ATR tablet) 3022 cm^{-1} [v(Ar-H)] 2922 cm^{-1} [v(CH$_3$)]2853 cm^{-1} [v(CH$_2$)] 1870 cm^{-1} [$v(\pi)$] that is exocyclic C═C vibration, 1559 cm^{-1} [v(C═N)], 1452 cm^{-1} [v(C═C bending)], 1209 cm^{-1} [v(C−C bending)], 752 cm^{-1} [v(C−H bending)] and 3401 and 1616 cm^{-1} is due to the O−H stretching and bending of absorbed water.

^{1}H NMR (CDCl$_3$-δ = 7.5 ppm) δ = 0.75 ppm (t, 6H), 1.5 (m, 4H), 2.0 (m, 20H), 3.6 (q, 4H), 4.5 (t, 4H), 5.2 (s, 2H), 7.5 (q, 4H), 8.6 (d, 2H), 10.2 (d, 2H), 10.75 (t, 4H).

^{13}C NMR (CDCl$_3$-δ = 80 ppm) δ = 13.97 ppm (CH$_3$), 22.51 (CH$_2$), 29.23 (CH$_2$), 30.1 (CH$_2$), 45.73 (CH$_2$), 47.83 (CH$_2$), 114.79 (CH$_2$ attached to positively charged nitrogen), 115.28 (CH), 127.6 (CH), 130.82 (C attached at the bridged position), 141.52 (CH═).

ESI-MS (LCMS): C$_{37}$H$_{58}$N$_4$Br$_2$ found 721, calcd 718; C$_{28}$H$_{39}$N$_4$Br$_2$ 557, calcd 585; C$_7$H$_{15}$ 110, calcd 110.7.

4.3. Physicochemical Characterization. The physicochemical characterization of two newly synthesized gemini

surfactants was done by using surface tensiometry (more details are given in the Supporting Information). The different interfacial parameters viz cmc, π_{cmc}, pC_{20}, Γ_{max}, A_{min}, and P were evaluated by using different equations and are given in Table 4.[10,16,17] In addition, the cmc values were also confirmed by UV-visible and pyrene probe fluorescence spectroscopy and given in the Supporting Information (Figure S9).

4.4. In Vitro Anti-candida Activity. Both the surfactants, BG8 and BG10 were investigated for their in vitro *anti-candida* activity against *C. albicans* (ATCC 90028), *C. glabrata* (ATCC 90030), *C. tropicalis* (ATCC 750), and FLC resistant *C. albicans* D15.9 strains by using the broth dilution procedure according to the quality protocol for antifungal assessment by NCCLS (National Committee for Clinical Laboratory Standards).[45] FLC (3.26 μM) was used as a positive control for the *anti-candida* study. The surfactant solutions were made in DMSO and diluted serially in a broth medium to attain the desired concentration of DMSO less than 1%. Variable concentrations of surfactants (1500−10.9 μM) were distributed into a 96 well plate in a Sabouraud dextrose (SD) broth medium in a 100 μL final volume. Further, 100 μL of yeast cells (approximate 2.5 × 10^3 cells/mL using (McFarland standard)[45] were distributed into the 96 well plate (Tarson) with incubation at 37 °C for 24 h. During the incubated period each well was investigated for the attendance or absence of visual growth of yeast cells. The lowermost concentration of the tested compound on which no observable growth occurs characterizes its MIC value. Moreover, the growth was observed turbidometrically at 600 nm using a ThermoMultiskan Go spectrophotometer after incubation.

4.5. Growth Curve Studies. The cells of *C. albicans* were revived freshly by a subculture on a SD agar plate. A loopful of inoculum was introduced into the SD broth, finally the cells were grown for 16 h at 37 °C before use. About 2 × 10^3 cells/mL were injected into the freshly prepared 50 mL sterile SD medium. Various concentrations equal to MIC, MIC/2, and 2MIC of the surfactants were separately added into the conical flasks comprising of the inoculated medium, which was incubated at 37 °C and 160 rpm. Strain specific concentration of FLC was used as positive control of 102 μM for *C. albicans* ATCC90028 and 3260 μM *C. albicans* D15.9 (FLC resistant), respectively. At prearranged time periods (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 after incubation with agitation at 37 °C), 1 mL aliquot from each sample in a conical flask was removed and growth was observed turbidometrically at 600 nm using a Thermo Multiskan spectrophotometer. The optical densities were recorded against each concentration with time (hour).

4.6. FIC Index/Synergistic Activity. The fractional inhibitory concentration (FIC) index or synergistic activity of BG8 and BG10 with FLC was determined against *C. albicans*. Synergy and antagonism were defined by FIC indices of <0.5 and >4, respectively. The FIC index result of > 0.5, but < 4 was considered indifferent. Both surfactants BG8 and BG10 were serially diluted in growth medium with concentration ranging from 95 to 0.04 μM against *C. albicans* ATCC90028 and 62.5−0.03 μM of FLC, respectively. The

MIC values of compound alone and in combination with FLC were determined by broth micro dilution method in 96 well plate. FIC index was calculated by using the following eq 1[41]

$$FIC = \frac{\text{MIC of drug A in combination with B}}{\text{MIC of drug A alone}}$$
$$+ \frac{\text{MIC of drug B in combination with A}}{\text{MIC of drug B alone}} \quad (1)$$

4.7. Ergosterol Biosynthesis in *C. albicans*. The whole intracellular sterols were estimated as reported earlier using standard *C. albicans* ATCC 90028. Three distinct conical flasks containing compounds BG8 and BG10 at MIC, MIC/2, and MIC/4 in the SD broth injected with freshly cultured cells of *C. albicans* ATCC 90028 were used. FLC (130 μM) was taken as the positive control and untreated cells were taken as the negative control for comparison and conical flasks were incubated at 35 °C for 16 h. The cells were harvested at their stationary phase after incubation and the weight of the pellet was determined. The pellet was treated with KOH solution (25% alcoholic potassium hydroxide) followed by incubation at 85 °C for 1 h. After incubation, sterol was removed by the addition of n-heptane/distilled water (1:3 ratio). The layers of heptane were moved into the fresh test tubes diluted five-fold in 100% ethanol and observed spectrophotometrically in the range of 240−300 nm. The existence of ergosterol and late-sterol intermediate DHE in the extracted samples shows a characteristic four peak curve.[46,47]

4.8. Assessment of *Anti-candida* Compound Toxicity and Efficacy in *G. mellonella*. Sixth instar larvae of the *G. mellonella* greater wax: moth (Live foods Direct Ltd. Sheffield, England) were kept in the dark at 15 °C to avoid pupation. Larvae which weigh 0.22 ± 0.03 g were selected and used within 2 weeks of receiving. 10 healthy larvae per treatment and controls (n = 3) were positioned in sterile 9 cm Petri dishes creased with Whatman filter paper with some wood shavings. Larvae were adjusted to 30 °C for 1 h prior to all experiments and incubated at 30 °C for all studies. All experiments were done independently at three distinct times. For toxicity studies, larvae were injected with 20 μL of BG8 and BG10 (3030−76 μM) by last left pro-leg, and reaction to stimuli was measured every 24 h. To assess the antifungal efficacy of BG8 and BG10 a culture of *Candida albicans* were grown to the immobile phase (nearly 2 × 10^8/mL) in the YEPD broth (2% w/v glucose, 2% w/v bactopptone, 1% w/v yeast extract) at 30 °C and 200 rpm. By centrifugation, (2056g for 5 min) the cells were harvested by using a GS-6 Beckmann bench-centrifuge and washed in phosphate-buffered saline (PBS) three times and resuspended again in PBS at 5 × 10^5/20 μL. The larvae were injected with yeast cells via the last left pro-leg into the hemocoel with a Myjector U-100 insulin syringe (Terumo. Europe. N.V., Belgium) and positioned at 30 °C in the dark. One hour post injection, larvae were injected with compounds BG8 and BG10 at varying concentrations resuspended in PBS added with 10% DMSO (v/v) through the last right pro-leg. Infected larvae were injected with 20 μL PBS supplemented with 10% DMSO (v/v). To assess larval viability, larvae which did not respond to touch stimulus were considered dead.

4.9. Hemolytic Assay. The haemolytic activity of the surfactants BG8 and BG10 and the conventional antifungal drug FLC were determined on human red blood cells (hRBCs). Human erythrocytes from healthy individuals were collected in tubes containing EDTA as an anticoagulant. The erythrocytes were harvested by centrifugation for 10 min at 2000 rpm and 20°C, and washed three times in phosphate buffered saline (PBS). To the pellet, PBS was added to yield a 10% (v/v) erythrocytes/PBS suspension. The 10% suspension of erythrocytes was then further diluted with PBS at a 1:10 ratio. 100 μL of the final diluted erythrocytes was added to 100 μL of PBS having a previously determined concentration gradient (38 μM to 1.51 mM) of the test compounds in microcentrifuge tubes. Total hemolysis was attained in 1% Triton X-100. The tubes were incubated for 1 h at 37 °C and then centrifuged for 10 min at 2000 rpm at room temperature. From the supernatant fluid, 150 μL was transferred to a flat bottomed microtiter plate (Tarson), and the absorbance was measured spectrophotometrically at 450 nm by using a Thermo Multiskan spectrophotometer. The hemolysis percentage was calculated by following eq 2[36]

$$\% \text{ hemolysis} = \left[\frac{\begin{array}{c}(A_{450} \text{ of test compound treated sample} - A_{450} \\ \text{of buffer treated sample})\end{array}}{\begin{array}{c}(A_{450} \text{ of 1\% Triton X-100 treated sample} - A_{450} \\ \text{of buffer treated sample})\end{array}} \right]$$
$$\times 100\% \quad (2)$$

4.10. Cytotoxicity by MTT Assay. HEK293 cell lines were procured from the National Centre for Cell Sciences (NCCS) Pune, India. Dulbecco's modified Eagle's medium (DMEM), antibiotic cocktail, and fetal bovine serum (FBS) were procured from Gibco Life Technologies. Thermo Fisher Scientific (USA). MTT and trypsin-EDTA solution has been purchased from Sigma (St. Louis, MO). HEK293 cells has been cultured and maintained in DMEM media enriched with 10% heat inactivated FBS and 1% penicillin, streptomycin solution at 37 °C in a humidified atmosphere of 5% CO$_2$. Cell cultures have been routinely preserved and trypsinized not beyond 30 passages. HEK293 cells were broadcasted in triplicate in 96-well plate containing a cell count of approximately 9000−10 000 cells/well and incubated in a CO$_2$ incubator for 24 h. Then, the cells were incubated with increasing concentrations of test compounds (5−250 μM) in a final volume of 200 μL for 48 h at 37 °C in a CO$_2$ incubator. The mixture of the culture medium and compounds were removed after 48 h of incubation at 37 °C and cells have been washed two times with a PBS (pH 7.4) solution. After that, freshly prepared 20 μL MTT at a concentration of 48.27 mM in PBS and 100 μL of DMEM has been added to each well and the plates were incubated for 4−5 h at 37 °C in the CO$_2$ incubator. DMSO (150 μL/well) was added to solubilize the formazan crystals, the metabolized MTT product, and was allowed a short incubation of 10 min at 27 °C. The absorbance was noted at 570 nm on the multi-plate ELISA reader (Bio-Rad, USA). % viability was taken as the comparative absorbance of treated versus untreated control cells and plotted as a function of concentration of compounds. All the results were obtained in triplicate.

4.11. Statistical Analysis. The data were evaluated as one-way analysis of variance (AVOVA) to detect the mean values observed for control and after treatment with compounds. Dunnett's test was used to compare the treatment, control and statistical significance was set at $P \le 0.01$.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: mabid@jmi.ac.in (M.A.).
*E-mail: rpatel@jmi.ac.in. Phone: +91 8860634100. Fax: +91 11 26983409 (R.P.).

ORCID ⊕

Mohammad Abid: 0000-0002-0507-8451
Rajan Patel: 0000-0002-3811-2898

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Samosorn, S.; Tanwirat, B.; Muhamad, N.; Casadei, G.; Tomkiewicz, D.; Lewis, K.; Suksamrarn, A.; Prammananan, T.; Gornall, K. C.; Beck, J. L.; Bremner, J. B. Antibacterial activity of berberine-NorA pump inhibitor hybrids with a methylene ether linking group. *Bioorg. Med. Chem.* **2009**, *17*, 3866−3872.

(2) Brown, G. D.; Denning, D. W.; Levitz, S. M. *Tackling Human Fungal Infections*; American Association for the Advancement of Science, 2012.

(3) Lai, C.-C.; Tan, C.-K.; Huang, Y.-T.; Shao, P.-L.; Hsueh, P.-R. Current challenges in the management of invasive fungal infections. *J. Infect. Chemother.* **2008**, *14*, 77.

(4) Pfaller, M. A.; Diekema, D. J.; Rinaldi, M. G.; Barnes, R.; Hu, B.; Veselov, A. V.; Tiraboschi, N.; Nagy, E.; Gibbs, D. L. Results from the ARTEMIS DISK Global Antifungal Surveillance Study: a 6.5-year analysis of susceptibilities of Candida and other yeast species to fluconazole and voriconazole by standardized disk diffusion testing. *J. Clin. Microbiol.* **2005**, *43*, 5848−5859.

(5) Paiva, J. A.; Pereira, J. M. New antifungal antibiotics. *Curr. Opin. Infect. Dis.* **2013**, *26*, 168−174.

(6) Patel, R.; Mir, M. U. H.; Maurya, J. K.; Singh, U. K.; Maurya, N.; Parray, M. u. d.; Khan, A. B.; Ali, A. Spectroscopic and molecular modelling analysis of the interaction between ethane-1,2-diyl bis(N,N-dimethyl-N-hexadecylammoniumacetoxy)dichloride and bovine serum albumin. *Luminescence* **2015**, *30*, 1233−1241.

(7) Sharma, T.; Dohare, N.; Kumari, M.; Singh, U. K.; Khan, A. B.; Borse, M. S.; Patel, R. Comparative effect of cationic gemini surfactant and its monomeric counterpart on the conformational stability and activity of lysozyme. *RSC Adv.* **2017**, *7*, 16763−16776.

(8) Bhadani, A.; Singh, S. Synthesis and properties of thioether spacer containing gemini imidazolium surfactants. *Langmuir* **2011**, *27*, 14033−14044.

(9) Menger, F. M.; Keiper, J. S. Gemini surfactants. *Angew. Chem., Int. Ed.* **2000**, *39*, 1906−1920.

(10) Patel, R.; Khan, A. B.; Dohare, N.; Maroof Ali, M.; Rajor, H. K. Mixed micellization and interfacial properties of ionic liquid-type imidazolium gemini surfactant with amphiphilic drug amitriptyline hydrochloride and its thermodynamics. *J. Surfactants Deterg.* **2015**, *18*, 719−728.

(11) Leclercq, L.; Noujeim, N.; Schmitzer, A. R. Development ofN,N′-Diaromatic Diimidazolium Cations: Arene Interactions for Highly Organized Crystalline Materials. *Cryst. Growth Des.* **2009**, *9*, 4784−4792.

(12) Kim, S. S.; Zhang, W.; Pinnavaia, T. J. Ultrastable mesostructured silica vesicles. *Science* **1998**, *282*, 1302−1305.

(13) Tieke, B. Polymerisation of styrene in microemulsion with catanionic surfactant mixtures. *Colloid Polym. Sci.* **2005**, *283*, 421−430.

(14) Zana, R. Dimeric and oligomeric surfactants. Behavior at interfaces and in aqueous solution: a review. *Adv. Colloid Interface Sci.* **2002**, *97*, 205−253.

(15) Kamboj, R.; Singh, S.; Bhadani, A.; Kataria, H.; Kaur, G. Gemini imidazolium surfactants: synthesis and their biophysiochemical study. *Langmuir* **2012**, *28*, 11969−11978.

(16) Maurya, J. K.; Mir, M. U. H.; Singh, U. K.; Maurya, N.; Dohare, N.; Patel, S.; Ali, A.; Patel, R. Molecular investigation of the interaction between ionic liquid type gemini surfactant and lysozyme: A spectroscopic and computational approach. *Biopolymers* **2015**, *103*, 406−415.

(17) Wani, F. A.; Khan, A. B.; Alshehri, A. A.; Malik, M. A.; Ahmad, R.; Patel, R. Synthesis, characterization and mixed micellization study of benzene sulphonate based gemini surfactant with sodium dodecyl sulphate. *J. Mol. Liq.* **2019**, *285*, 270−278.

(18) Patel, R.; Mir, M. U. H.; Singh, U. K.; Beg, I.; Islam, A.; Khan, A. B. Refolding of urea denatured cytochrome c : Role of hydrophobic tail of the cationic gemini surfactants. *J. Colloid Interface Sci.* **2016**, *484*, 205−212.

(19) ud din Parray, M.; Maurya, N.; Wani, F.; Ahmad Wani, M. S.; Arfin, N.; Ahmad Malik, M.; Patel, R. Comparative effect of cationic gemini surfactant and its monomeric counterpart on the conformational stability of phospholipase A2. *J. Mol. Struct.* **2019**, *1175*, 49−55.

(20) Matsuoka, K.; Chiba, N.; Yoshimura, T.; Takeuchi, E. Effect of double quaternary ammonium groups on micelle formation of partially fluorinated surfactant. *J. Colloid Interface Sci.* **2011**, *356*, 624−629.

(21) Pei, X.; Zhao, J.; Wei, X. Wormlike micelles formed by mixed cationic and anionic gemini surfactants in aqueous solution. *J. Colloid Interface Sci.* **2011**, *356*, 176−181.

(22) Martins, L. M. S.; Mamizuka, E. M.; Carmona-Ribeiro, A. M. Cationic vesicles as bactericides. *Langmuir* **1997**, *13*, 5583−5587.

(23) Wong, Y.-L.; Hubieki, M. P.; Curfman, C. L.; Doncel, G. F.; Dudding, T. C.; Savle, P. S.; Gandour, R. D. A structure-activity study of spermicidal and anti-HIV properties of hydroxylated cationic surfactants. *Bioorg. Med. Chem.* **2002**, *10*, 3599−3608.

(24) Maisuria, B. B.; Actis, M. L.; Hardrict, S. N.; Falkinham, J. O.; Cole, M. F.; Cihlar, R. L.; Peters, S. M.; Macri, R. V.; Sugandhi, E. W.; Williams, A. A.; Poppe, M. A.; Esker, A. R.; Gandour, R. D. Comparing micellar, hemolytic, and antibacterial properties of di- and tricarboxyl dendritic amphiphiles. *Bioorg. Med. Chem.* **2011**, *19*, 2918−2926.

(25) Ding, M.; He, X.; Wang, Z.; Li, J.; Tan, H.; Deng, H.; Fu, Q.; Gu, Q. Cellular uptake of polyurethane nanocarriers mediated by gemini quaternary ammonium. *Biomaterials* **2011**, *32*, 9515−9524.

(26) Falsini, S.; Ristori, S.; Ciani, L.; Di Cola, E.; Supuran, CT; Arcangeli, A.; In, M. Time resolved SAXS to study the complexation of siRNA with cationic micelles of divalent surfactants. *Soft Matter* **2014**, *10*, 2226−33.

(27) In, S.; Ciani, L.; Candiani, G.; Battistini, C.; Frati, A.; Grillo, I.; In, M. Complexing a small interfering RNA with divalent cationic surfactants. *Soft Matter* **2012**, *8*, 749−756.

(28) Muzzalupo, R.; Infante, M. R.; Pérez, L.; Pinazo, A.; Marques, E. F.; Antonelli, M. L.; Strinati, C.; La Mesa, C. Interactions between gemini surfactants and polymers: Thermodynamic studies. *Langmuir* **2007**, *23*, 5963−5970.

(29) Brycki, B.; Szulc, A. Gemini alkyldeoxy-D-glucitolammonium salts as modern surfactants and microbiocides: synthesis, antimicrobial and surface activity, biodegradation. *PLoS One* **2014**, *9*, No. e84936.

(30) Obłąk, E.; Piecuch, A.; Krasowska, A.; Łuczyński, J. Antifungal activity of gemini quaternary ammonium salts. *Microbiol. Res.* **2013**, *168*, 630−638.

(31) Tan, H.; Xiao, H. Synthesis and antimicrobial characterization of novel l-lysine gemini surfactants pended with reactive groups. *Tetrahedron Lett.* **2008**, *49*, 1759−1761.

(32) Ruiz, A.; Pinazo, A.; Pérez, L.; Manresa, A.; Marqués, A. M. Green Catanionic Gemini Surfactant-Lichenysin Mixture: Improved Surface, Antimicrobial, and Physiological Properties. *ACS Appl. Mater. Interfaces* **2017**, *9*, 22121−22131.

(33) Zhang, Y.; Ding, M.; Zhou, L.; Tan, H.; Li, J.; Xiao, H.; Li, J.; Snow, J. Synthesis and antibacterial characterization of gemini surfactant monomers and copolymers. *Polym. Chem.* **2012**, *3*, 907−913.

(34) Paluch, E.; Piecuch, A.; Obłąk, E.; Lamch, Ł.; Wilk, K. A. Antifungal activity of newly synthesized chemodegradable dicephalic-type cationic surfactants. *Colloids Surf., B* **2018**, *164*, 34−41.

(35) Piecuch, A.; Obłąk, E.; Guz-Regner, K. Antibacterial Activity of Alanine-Derived Gemini Quaternary Ammonium Compounds. *J. Surfactants Deterg.* **2016**, *19*, 275−282.

(36) Masood, M. M.; Irfan, M.; Khan, P.; Alajmi, M. F.; Hussain, A.; Garrison, J.; Rehman, M. T.; Abid, M. 1,2,3-Triazole-quinazolin-4(3H)-one conjugates: evolution of ergosterol inhibitor as anti-candidal agent. *RSC Adv.* **2018**, *8*, 39611−39625.

(37) Aneja, B.; Azam, M.; Alam, S.; Perwez, A.; Maguire, R.; Yadava, U.; Kavanagh, K.; Daniliuc, C. G.; Rizvi, M. M. A.; Haq, Q. M. R.; Abid, M. Natural Product-Based 1,2,3-Triazole/Sulfonate Analogues as Potential Chemotherapeutic Agents for Bacterial Infections. *ACS Omega* **2018**, *3*, 6912−6930.

(38) Rowan, R.; Moran, C.; McCann, M.; Kavanagh, K. Use of Galleria mellonella larvae to evaluate the in vivo anti-fungal activity of [Ag2(mal)(phen)3]. *BioMetals* **2009**, *22*, 461.

(39) Browne, N.; Hackenberg, F.; Streciwilk, W.; Tacke, M.; Kavanagh, K. Assessment of in vivo antimicrobial activity of the carbene silver(I) acetate derivative SBC3 using Galleria mellonella larvae. *BioMetals* **2014**, *27*, 745−752.

(40) McCann, M.; Santos, A. L. S.; Da Silva, B. A.; Romanos, M. T. V.; Pyrrho, A. S.; Devereux, M.; Kavanagh, K.; Fichtner, I.; Kellett, A. In vitro and in vivo studies into the biological activities of 1,10-phenanthroline, 1,10-phenanthroline-5,6-dione and its copper(ii) and silver(i) complexes. *Toxicol. Res.* **2012**, *1*, 47−54.

(41) Irfan, M.; Alam, S.; Manzoor, N.; Abid, M. Effect of quinoline based 1,2,3-triazole and its structural analogues on growth and virulence attributes of Candida albicans. *PLoS One* **2017**, *12*, No. e0175710.

(42) Reddy, A. R. N.; Reddy, Y. N.; Krishna, D. R.; Himabindu, V. Multi wall carbon nanotubes induce oxidative stress and cytotoxicity in human embryonic kidney (HEK293) cells. *Toxicology* **2010**, *272*, 11−16.

(43) Su, Y.; Hu, M.; Fan, C.; He, Y.; Li, Q.; Li, W.; Wang, L.-h.; Shen, P.; Huang, Q. The cytotoxicity of CdTe quantum dots and the relative contributions from released cadmium ions and nanoparticle properties. *Biomaterials* **2010**, *31*, 4829−4834.

(44) Selvaraj, V.; Bodapati, S.; Murray, E.; Blough, E.; Winston, N.; Rice, K.; Shokufar, T.; Zhao, Y. Cytotoxicity and genotoxicity caused by yttrium oxide nanoparticles in HEK293 cells. *Int. J. Nanomed.* **2014**, *9*, 1379.

(45) Wayne, P. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, Approved Standard*; CLSI document M27-A2, 2002.

(46) Aneja, B.; Irfan, M.; Kapil, C.; Jairajpuri, M. A.; Maguire, R.; Kavanagh, K.; Rizvi, M. M. A.; Manzoor, N.; Azam, A.; Abid, M. Effect of novel triazole-amino acid hybrids on growth and virulence of Candida species: in vitro and in vivo studies. *Org. Biomol. Chem.* **2016**, *14*, 10599−10619.

(47) Arthington-Skaggs, B. A.; Warnock, D. W.; Morrison, C. J. Quantitation of Candida albicans Ergosterol Content Improves the Correlation between In Vitro Antifungal Susceptibility Test Results and In Vivo Outcome after Fluconazole Treatment in a Murine Model of Invasive Candidiasis. *Antimicrob. Agents Chemother.* **2000**, *44*, 2081−2085.