European Journal of Medicinal Chemistry 133 (2017) 309–318

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Novel alkylated azoles as potent antifungals

Sanjib K. Shrestha [1], Atefeh Garzan [1], Sylvie Garneau-Tsodikova*

Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 789 South Limestone Street, Lexington, KY 40536-0596, USA

ARTICLE INFO

Article history:
Received 18 January 2017
Received in revised form
27 March 2017
Accepted 30 March 2017
Available online 31 March 2017

Keywords:
Cytotoxicity
Ergosterol
Fluconazole
Hemolysis
Time-kill curves

ABSTRACT

Fluconazole (FLC) is the drug of choice when it comes to treat fungal infections such as invasive candidiasis in humans. However, the widespread use of FLC has resulted in the development of resistance to this drug in various fungal strains and, simultaneously has occasioned the need for new antifungal agents. Herein, we report the synthesis of 27 new FLC derivatives along with their antifungal activity against a panel of 13 clinically relevant fungal strains. We also explore their toxicity against mammalian cells, their hemolytic activity, as well as their mechanism of action. Overall, many of our FLC derivatives exhibited broad-spectrum antifungal activity and all compounds displayed an MIC value of <0.03 μg/mL against at least one of the fungal strains tested. We also found them to be less hemolytic and less cytotoxic to mammalian cells than the FDA approved antifungal agent amphotericin B. Finally, we demonstrated with our best derivative that the mechanism of action of our compounds is the inhibition of the sterol 14α-demethylase enzyme involved in ergosterol biosynthesis.

1. Introduction

Fungal infections have been rapidly increasing worldwide and present a continuous threat to human health [1]. Drug resistance among fungal pathogens is an increasing problem, thus identification and development of compounds capable of overcoming resistance is a requisite [2]. The conventional antifungal agents used in the treatment of human fungal infections are azoles (*e.g.*, fluconazole (FLC), voriconazole (VOR), itraconazole (ITC), and posaconazole (POS)), polyenes (*e.g.*, amphotericin B (AmB)) (Fig. 1), echinocandins (*e.g.*, anidulafungin, caspofungin (CAS), and micafungin), and allylamines (*e.g.*, terbinafine and naftifine) [3]. We previously showed that kanamycin B (KANB) and tobramycin (TOB) analogues with linear alkyl chains comprising 12 and 14 carbons (C_{12} and C_{14}; Fig. 1) display promising antifungal potency against *Candida albicans* and *Aspergillus* spp. [4,5]. Unlike the parent aminoglycoside antibiotics, the C_{12} and C_{14} KANB and TOB analogues appear to inhibit fungi by disrupting the fungal membrane as a novel mechanism of action. Similarly, we recently demonstrated that *n*-alkylated ebsulfur derivatives, especially that containing a C_5 alkyl chain, display strong antifungal activity, albeit without disrupting the fungal membrane [6,7]. Currently, azoles have been used with considerable success in the treatment of serious fungal infections due to their high therapeutic index, their favorable pharmacokinetic (PK) parameters, excellent activity against *Candida* spp., and good safety profile [3]. One of the most important members of the azoles family is FLC, which was one of the first azole drugs to contain a quaternary center comprising an hydroxyl moiety [8]. FLC has been widely applied clinically. However, it is not effective against invasive aspergillosis and the number of FLC-resistant strains has augmented significantly with the increased use of this antifungal agent [9,10]. ITC, one of the other azoles, shows stronger activity against *Aspergillus* spp. than does FLC, but has poor aqueous solubility and oral bioavailability [11]. Many novel azoles have been developed to overcome these disadvantages, including second-generation azoles such as VOR, POS, ravuconazole, isavuconazole, and albaconazole, which demonstrate favorable antifungal activity, improved PK properties, and acceptable toxicity profiles [12]. Some of these newer azole derivatives (*e.g.*, VOR, POS, ITC, ravuconazole, and isavuconaozle) were generated by replacing one of the triazole rings of FLC by other moieties. An azole drug, hexaconazole (labeled compound **27** in Fig. 2 of this study) comprising a C_4 alkyl chain in its structure, is an FDA-approved agrifungicide used to treat fungal infection in agriculture [13,14]. Some types of fungal infections such as pulmonary infections with *Aspergillus* can cause swallowing difficulties, so there is a pressing medical need for injectable antifungal agents [15]. Herein, inspired by the clinical applicability of azoles, the use of hexaconazole as a fungicidal agent, and the promise of our C_{12}

* Corresponding author.
E-mail address: sylviegtsodikova@uky.edu (S. Garneau-Tsodikova).
[1] These two authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ejmech.2017.03.075
0223-5234/

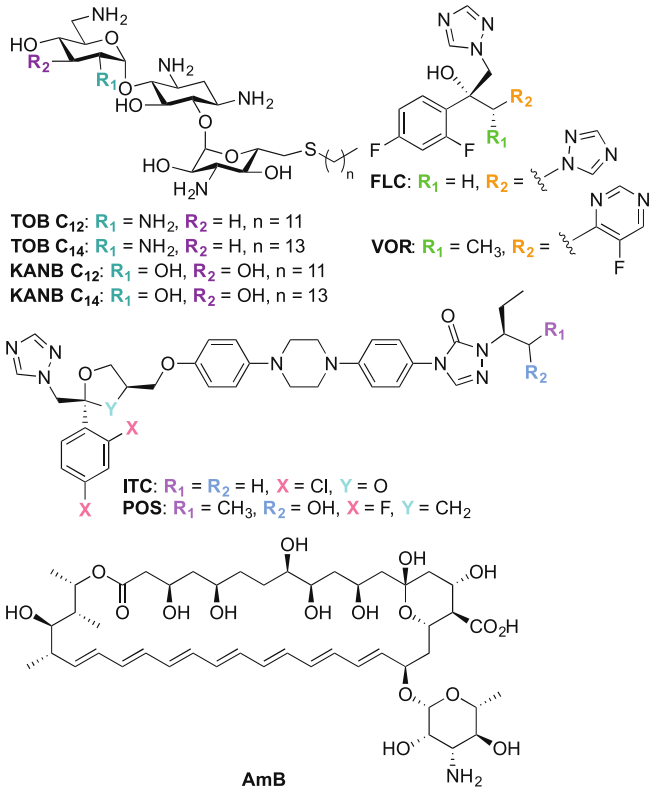


Fig. 1. Structures of compounds discussed in this study.

and C_{14} KANB and TOB analogues and C_5 ebsulfur derivative, we decided to introduce a linear alkyl chain in place of one of the triazole rings of FLC and vary the halide groups on its phenyl ring to see if we could generate FLC derivatives with improved antifungal activity. We also investigated time-kill and hemolysis activities of these compounds, as well as cytotoxicity against mammalian cells. Finally, we explored and established potential mechanisms of action for our new FLC derivatives.

2. Results and discussion

2.1. Design and chemical synthesis of compounds *1-41*

The synthetic pathways for the preparation of target compounds **1**–**9** and **24**–**41** are depicted in Fig. 2. We synthesized compounds **1**–**9** using a straightforward strategy, which involved the Grignard reactions of 2,4-difluoro-α-(1*H*-1,2,4-triazolyl)acetophenone with different alkyl magnesium bromide in the presence of magnesium bromide ethyl etherate. Compounds **24**–**41** were prepared in three steps. 4-Amino-1,2,4-triazole was reacted with various 2-haloacetophenone in refluxing isopropanol to give **10**–**16** in excellent yields (88–96%). Compounds **10**–**16** were conveniently deaminated by $NaNO_2$ in aqueous HCl at room temperature. The desired products **17**–**23** were precipitated after neutralization of the reaction with potassium carbonate. The precipitates were collected by filtration and washed with water to afford pure products. No further purification was required because the excess of reactants was soluble in water and removed by filtration. Compounds **17**–**23** were further converted to the corresponding products **24**–**41** by Grignard reaction in the presence of different alkyl magnesium bromide.

With these compounds in hand, we aimed to answer the six following questions in terms of structure-activity-relationship (SAR) (*Note*: We put the identity of the compounds used to answer these questions into parentheses after the questions): (i) what is/are the optimal length(s) of the newly added alkyl chains to confer antifungal activity? (compounds **1**–**9**); (ii) are these optimal chain lengths for compounds **1**–**9** the same as that of other families of *n*-alkylated molecules (*e.g.*, aminoglycoside, benzimidazole, and ebsulfur derivatives)?; (iii) for a given alkyl chain length, would a 2,4-dichlorinated phenyl ring (compounds **24**–**29**) confer better or worse antifungal activity than the 2,4-difluorinated phenyl ring (compounds **1**–**6**)?; (iv) for a mono-substituted phenyl ring, which halogen substituent is best? (compounds **30**–**35** *versus* their counterparts **36**–**41**); (v) for a specific alkyl chain length, which level of substitution (mono- *versus* di-) confer the best antifungal activity? (compound **5** *versus* compounds **30**, **32**, and **34**; compound **6** *versus* compounds **31**, **33**, and **35**; compound **28** *versus* compounds **36**, **38**, and **40**; compound **29** *versus* compounds **37**, **39**, and **41**); and (vi) for a given substituent, what is the optimal position (*ortho*, *meta*, or *para*) for mono-substitution on the phenyl ring? (compounds **30** *versus* **32** *versus* **34**; compounds **31** *versus* **33** *versus* **35**; compounds **36** *versus* **38** *versus* **40**; compounds **37** *versus* **39** *versus* **41**).

2.2. Antifungal activity

The antifungal activity of our new azole compounds **1**–**9** and **24**–**41 as well as that of intermediate 10** was first evaluated against a panel of seven *Candida albicans* strains (**A-G**), three non-*albicans Candida* strains (**H-**–**J**), and three *Aspergillus* strains (**K-M**) in a concentration range of 0.03–31.3 μg/mL (Table 1). Out of the *C. albicans* strains, two were classified as sensitive (strains **C** and **E**), one as intermediate (strain **A**), and four as resistant (strains **B**, **D**, **F**, and **G**) to FLC and ITC as defined by the American Type Culture Collection (ATCC). For all of our azole derivatives we report their MIC-0 values against *C. albicans* ATCC 10231 (strain **A**), *C. krusei* (strain **I**), *C. parapsilosis* (strain **J**), and all *Aspergillus* strains (**K-M**), which correspond to complete growth inhibition (fungicidal activity) of these fungi. However, as all other *Candida* strains tested (**B-**–**H**) display a trailing growth effect (indicating that the compounds are fungistatic), we report the MIC-2 values of our derivatives against these strains, which correspond to 50% growth inhibition of these fungal strains. The commercially available antifungals AmB, CAS, FLC, ITC, POS, and VOR were used as reference drugs for comparison. The MIC values presented for these six control drugs were either tested herein (CAS and VOR) or correspond to our previously published data (AmB, FLC, ITC, and POS) [5,16]. The antifungal activity of one of our intermediates, the amino-triazole **10**, was also determined to confirm that the intermediates generated during the synthesis of our final derivatives do not exert any antifungal activity against the fungal strains tested. From here on, we designate antifungal activity as either excellent (<0.03–1.95 μg/mL), moderate (3.9–7.8 μg/mL), or poor (15.6-≥31.3 μg/mL) based on MIC values.

By performing a broad survey of the MIC data presented in Table 1, we rapidly could identify the following general trends. The three non-*albicans Candida* strains (strains **H-**–**j**) tested were extremely susceptible to the majority of our compounds with strain **J** being the most susceptible, followed by strain **I** and then by strain **H**. Likewise, most of our compounds displayed excellent antifungal activity against two of the three *Aspergillus* spp. tested (strains **L** and **M**). However, against *Aspergillus flavus* ATCC MYA-3631 (strain **K**) only compounds **27**–**30** and **37**–**38** displayed moderate antifungal activity. When focusing on the *C. albicans* strains, we observed that our compounds were very effective against the majority of these strains (**A**, **C-G**), with the exception of the azole-resistant strain **B**. In fact, against strain **B** only two compounds

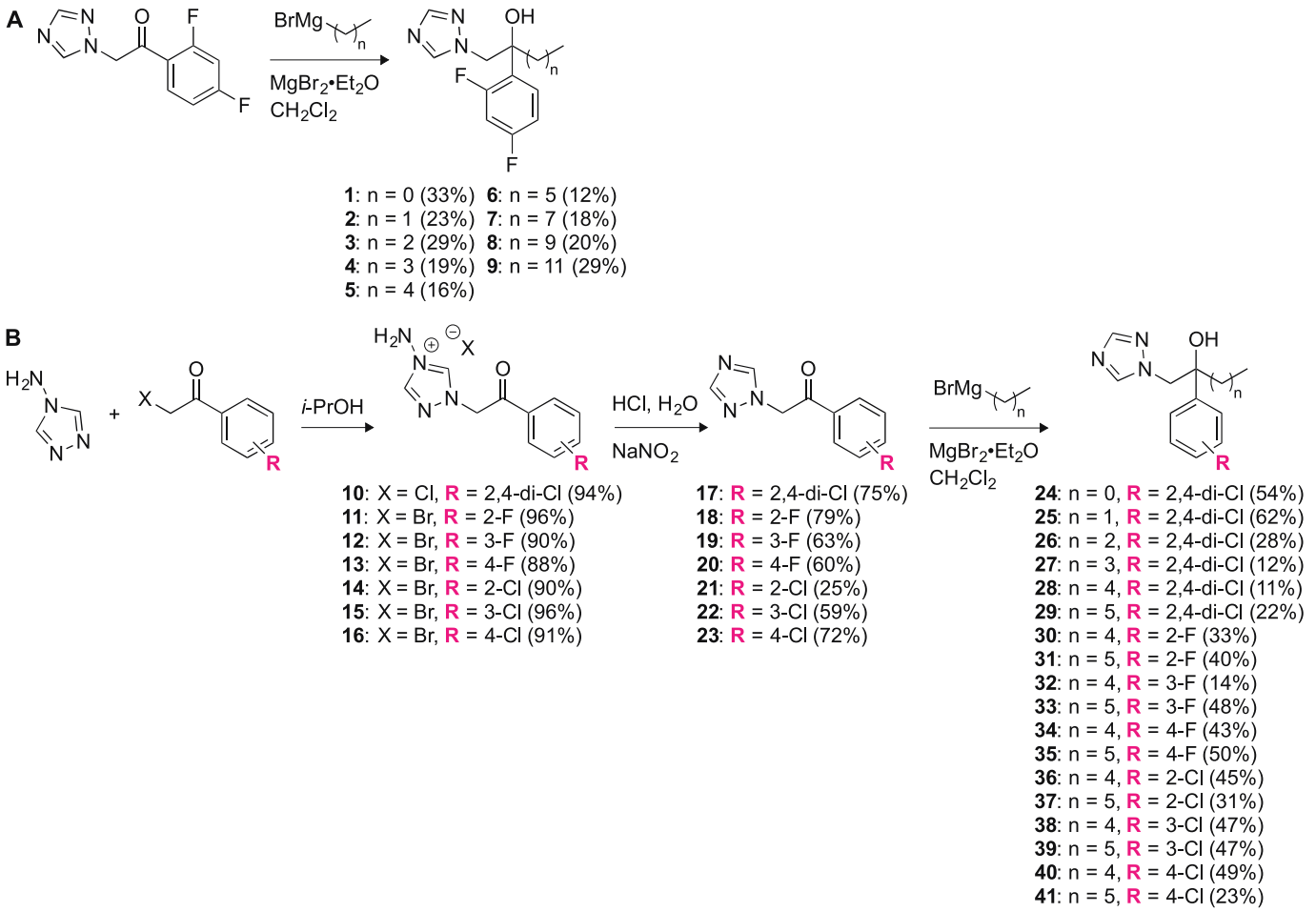


Fig. 2. Synthetic schemes for the preparation of **A.** compounds **1**–**9** and **B.** compounds **24**–**41**.

(**28** and **29**) showed excellent antifungal activity and six compounds (**5**, **6**, **7**, **26**, **27**, and **40**) displayed moderate antifungal activity. It is noteworthy to point out that most of our compounds maintained a high degree of activity against most of the azole-resistant strains of *C. albicans* tested. From these general observations, we identified compounds **28** and **29** to be our best FLC derivatives based on their excellent antifungal activity against 11 fungal strains, and moderate activity against 2 fungal strains out of all 13 strains evaluated. In addition, six compounds stood out (**7**, **27**, **30**, **31**, **36**, and **37**) as they showed good to moderate antifungal activity against 10–13 fungal strains. It was promising to observe that only three compounds (**1**, **2**, and **39**) showed poor antifungal activity.

2.3. SAR analysis

In concordance with our antifungal results discussed above (Table 1), we approached the eight questions raised at the beginning of this study. (i) What is/are the optimal length(s) of the newly added alkyl chains to confer antifungal activity? In order to decipher which chain length confers optimal antifungal activity, we synthesized nine compounds (**1**–**9**) by replacing one of the two triazole rings of FLC with linear alkyl chains varying in length from 1 to 12 carbons (C_1-C_{12}) (Table 1). When analyzing the MIC data in Table 1, we observed that compounds **1**–**4** with shorter C_1-C_4 alkyl substituents and compounds **8** and **9** with longer C_{10} and C_{12} alkyl moieties were in general not great at stopping fungal cell growth. However, compounds **5**–**7** with alkyl chains of medium lengths, C_5, C_6, and C_8, were in general great antifungals. When carefully analyzing the MIC data presented in Table 1, we determined that in most cases, compounds **1** and **2** with the two shortest alkyl chains exhibited the worse antifungal profile with most MIC values varying between 15.6 and ≥31.3 μg/mL against 10 of the 13 fungal strains tested (6 *C. albicans* strains (**A-F**), *C. glabrata* ATCC 2001 (**H**), and 3 *Aspergillus* strains (**K-M**)). In addition, compound **1** with a methyl substituent also displayed poor antifungal activity (MIC = 15.6-≥31.3 μg/mL) against *C. albicans* ATCC MYA-1003 (strain **G**) and *C. krusei* ATCC 6258 (strain **I**). Actually compound **1** only displayed moderate activity against *C. parapsilosis* ATCC 22019 (strain **J**), whereas compound **2** displayed excellent activity (0.48 μg/mL) against this strain **J** and moderate activity (MIC = 7.8 μg/mL) against strains **G** and **I**. Likewise, compounds **3** and **4** with C_3 and C_4 alkyl chains were found to be poor antifungals (15.6->31.3 μg/mL) against 6 (strains **B**, **D**, **F**, **H**, **K**, and **M**) of the 13 strains tested. It is to note that the growth of these 6 strains was also not easily inhibited by compounds **1** and **2**. However, unlike compounds **1** and **2**, compounds **3** and **4** showed some improvement in terms of their antifungal activity against 4 *C. albicans* strains (**A**, **C**, **E**, and **G**) and *A. nidulans* ATCC 38163 (strain **L**). Compound **3** displayed moderate antifungal activity (3.9–7.8 μg/mL) against 3 of *C. albicans* strains (**A**, **E**, and **G**) and *A. nidulans* ATCC 38163 (strain **L**), whereas compound **4** displayed moderate activity only against *C. albicans* ATCC 64124 (strain **C**) and *C. albicans* ATCC MYA-1003 (strain **G**). On a good note, compounds **3** and **4** displayed

Table 1
MIC values[a] in μg/mL (*Note*: the corresponding MIC values in μM are presented into parentheses below the value in μg/mL) determined for compounds **1**–**9**, **24**–**41**, intermediate **10**, and for six control antifungal agents (AmB, CAS, FLC, ITC, POS, and VOR) against various yeast strains and filamentous fungi.

Cpd#	Yeast strains										Filamentous fungi		
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**
1	>31.3 (>130.8)	>31.3 (>130.8)	>31.3 (>130.8)	>31.3 (>130.8)	>31.3 (>130.8)	>31.3 (>130.8)	15.6 (65.2)	>31.3 (>130.8)	>31.3 (>130.8)	3.9 (16.3)	>31.3 (>130.8)	31.3 (130.8)	>31.3 (>130.8)
2	15.6 (61.6)	>31.3 (>123.6)	15.6 (61.6)	31.3 (123.6)	>31.3 (>123.6)	>31.3 (>123.6)	7.8 (30.8)	>31.3 (>123.6)	7.8 (30.8)	0.48 (1.9)	>31.3 (>123.6)	31.3 (123.6)	>31.3 (>123.6)
3	3.9 (14.6)	31.3 (117.1)	15.6 (58.4)	15.6 (58.4)	7.8 (29.2)	31.3 (117.1)	3.9 (14.6)	>31.3 (>117.1)	0.06 (0.22)	0.06 (0.22)	>31.3 (>117.1)	7.8 (29.2)	15.6 (58.4)
4	1.95 (6.9)	15.6 (55.5)	7.8 (27.7)	15.6 (55.5)	31.3 (111.7)	15.6 (55.5)	3.9 (13.9)	31.3 (111.7)	0.06 (0.21)	<0.03 (<0.11)	15.6 (55.5)	1.95 (6.9)	15.6 (55.5)
5	1.95 (6.6)	7.8 (26.4)	7.8 (26.4)	3.9 (13.2)	7.8 (26.4)	7.8 (26.4)	1.95 (6.6)	7.8 (26.4)	0.24 (0.81)	<0.03 (<0.10)	15.6 (52.8)	0.975 (3.3)	>31.3 (>106.0)
6	1.95 (6.3)	7.8 (25.2)	1.95 (6.3)	1.95 (6.3)	3.9 (12.6)	1.95 (6.3)	1.95 (6.3)	3.9 (12.6)	0.12 (0.39)	<0.03 (<0.10)	15.6 (50.4)	0.48 (1.6)	7.8 (25.2)
7	1.95 (5.8)	3.9 (11.6)	1.95 (5.8)	1.95 (5.8)	1.95 (5.8)	1.95 (5.8)	1.95 (5.8)	1.95 (5.8)	0.24 (0.71)	≤0.06 (≤0.18)	31.3 (92.8)	0.48 (1.4)	7.8 (23.1)
8	7.8 (21.3)	15.6 (42.7)	7.8 (21.3)	31.3 (85.6)	7.8 (21.3)	>31.3 (>85.6)	>31.3 (>85.6)	7.8 (21.3)	0.24 (0.66)	0.12 (0.33)	>31.3 (>85.6)	0.24 (0.66)	3.9 (10.7)
9	>31.3 (>79.5)	>31.3 (>79.5)	7.8 (19.8)	>31.3 (>79.5)	7.8 (19.8)	7.8 (19.8)	>31.3 (>79.5)	0.975 (2.5)	>31.3 (>79.5)	1.95 (5.0)	>31.3 (>79.5)	0.48 (1.2)	3.9 (9.9)
10	>31.3 (>101.8)	>31.3 (>101.8)	>31.3 (>101.8)	>31.3 (>101.8)	>31.3 (>101.8)	>31.3 (>101.8)	>31.3 (>101.8)	>31.3 (>101.8)	>31.3 (>101.8)	>31.3 (>101.8)	>31.3 (>101.8)	>31.3 (>101.8)	>31.3 (>101.8)
24	>31.3 (>115.0)	>31.3 (>115.0)	7.8 (28.7)	1.95 (7.2)	>31.3 (>115.0)	>31.3 (>115.0)	>31.3 (>115.0)	3.9 (14.3)	3.9 (14.3)	0.24 (0.88)	>31.3 (>115.0)	1.95 (7.2)	>31.3 (>115.0)
25	3.9 (13.6)	>31.3 (>109.4)	0.975 (3.4)	0.24 (0.84)	0.975 (3.4)	0.975 (3.4)	1.95 (6.8)	3.9 (13.6)	0.975 (3.4)	<0.03 (<0.10)	15.6 (54.5)	7.8 (27.3)	3.9 (13.6)
26	0.24 (0.8)	3.9 (13.0)	1.95 (6.5)	3.9 (13.0)	3.9 (13.0)	7.8 (26.0)	0.975 (3.2)	3.9 (13.0)	0.06 (0.20)	<0.03 (<0.10)	15.6 (52.0)	1.95 (6.5)	3.9 (13.0)
27	0.24 (0.76)	3.9 (12.4)	1.95 (6.2)	1.95 (6.2)	1.95 (6.2)	7.8 (24.8)	0.975 (3.1)	3.9 (12.4)	0.06 (0.19)	<0.03 (<0.10)	3.9 (12.4)	0.24 (0.76)	0.975 (3.1)
28	0.24 (0.73)	1.95 (5.9)	0.975 (3.0)	1.95 (5.9)	1.95 (5.9)	1.95 (5.9)	0.975 (3.0)	1.95 (5.9)	0.48 (1.5)	<0.03 (<0.09)	3.9 (11.9)	0.48 (1.5)	3.9 (11.9)
29	0.24 (0.70)	1.95 (5.7)	0.975 (2.8)	1.95 (5.7)	1.95 (5.7)	1.95 (5.7)	0.975 (2.8)	1.95 (5.7)	0.03 (0.09)	<0.03 (<0.09)	3.9 (11.4)	0.48 (1.4)	3.9 (11.4)
30	3.9 (14.1)	>31.3 (>112.9)	0.975 (3.5)	0.48 (1.7)	0.975 (3.5)	0.975 (3.5)	3.9 (14.1)	0.975 (3.5)	0.06 (0.22)	0.12 (0.43)	7.8 (28.1)	0.48 (1.7)	3.9 (14.1)
31	1.95 (6.7)	>31.3 (>107.4)	0.975 (3.3)	0.24 (0.82)	0.975 (3.3)	0.975 (3.3)	3.9 (13.4)	0.975 (3.3)	0.06 (0.21)	0.12 (0.41)	15.6–31.3 (53.5–107.4)	0.48 (1.6)	3.9 (13.4)
32	>31.3 (>112.9)	>31.3 (>112.9)	3.9 (14.1)	3.9 (14.1)	3.9 (14.1)	3.9 (14.1)	>31.3 (>112.9)	7.8 (28.1)	3.9 (14.1)	3.9 (14.1)	≥31.3 (≥112.9)	0.24 (0.87)	3.9 (14.1)
33	>31.3 (>107.4)	>31.3 (>107.4)	1.95 (6.7)	1.95 (6.7)	1.95 (6.7)	0.975 (3.3)	31.3 (107.4)	7.8 (26.8)	3.9 (13.4)	1.95 (6.7)	>31.3 (>107.4)	3.9 (13.4)	31.3 (107.4)
34	3.9 (14.1)	>31.3 (>112.9)	7.8 (28.1)	0.975 (3.5)	3.9 (14.1)	1.95 (7.0)	>31.3 (>112.9)	7.8 (28.1)	0.975 (3.5)	0.48 (1.7)	>31.3 (>112.9)	3.9 (14.1)	31.3 (112.9)
35	15.6 (53.5)	>31.3 (>107.4)	7.8 (26.8)	0.975 (3.3)	7.8 (26.8)	31.3 (107.4)	7.8 (26.8)	7.8 (26.8)	0.975 (3.3)	0.48 (1.6)	>31.3 (>107.4)	1.95 (6.7)	15.6 (53.5)
36	7.8 (26.5)	>31.3 (>106.5)	0.975 (3.3)	0.48 (1.6)	0.975 (3.3)	0.975 (3.3)	7.8 (26.5)	0.975 (3.3)	0.975 (3.3)	0.12 (0.41)	>31.3 (>106.5)	0.975 (3.3)	15.6 (53.1)
37	7.8 (25.3)	>31.3 (>101.7)	0.975 (3.2)	1.95 (6.3)	1.95 (6.3)	0.975 (3.2)	7.8 (25.3)	0.975 (3.2)	0.12 (0.39)	0.12 (0.39)	1.95–3.9 (6.3–12.7)	0.06 (0.19)	0.48 (1.6)
38	>31.3 (>106.5)	31.3 (106.5)	>31.3 (>106.5)	3.9 (13.3)	1.95 (6.6)	1.95 (6.6)	>31.3 (>106.5)	>31.3 (>106.5)	>31.3 (>106.5)	>31.3 (>106.5)	3.9 (13.3)	0.06 (0.20)	0.48 (1.6)
39	>31.3 (>101.7)	31.3 (101.7)	>31.3 (>101.7)	7.8 (25.3)	7.8 (25.3)	7.8 (25.3)	>31.3 (>101.7)	>31.3 (>101.7)	>31.3 (>101.7)	>31.3 (>101.7)	>31.3 (>101.7)	3.9 (12.7)	>31.3 (>101.7)
40	1.95 (6.6)	7.8 (26.5)	15.6 (53.1)	1.95 (6.6)	15.6 (53.1)	3.9 (13.3)	1.95 (6.6)	3.9 (13.3)	0.12 (0.41)	0.06 (0.20)	>31.3 (>106.5)	7.8 (26.5)	>31.3 (>106.5)
41	1.95 (6.3)	31.3 (101.7)	7.8 (25.3)	3.9 (12.7)	31.3 (101.7)	3.9 (12.7)	1.95 (6.3)	>31.3 (>101.7)	1.95 (6.3)	>31.3 (>101.7)	>31.3 (>101.7)	3.9 (12.7)	15.6 (50.7)
AmB	3.9 (4.2)	3.9 (4.2)	1.95 (2.1)	0.975 (1.1)	1.95 (2.1)	3.9 (4.2)	3.9 (4.2)	1.95 (2.1)	3.9 (4.2)	1.95 (2.1)	15.6 (16.9)	15.6 (16.9)	3.9 (4.2)
CAS	0.975 (0.8)	0.24 (0.2)	0.06 (0.05)	0.12 (0.1)	0.12 (0.1)	0.24 (0.2)	0.48 (0.4)	0.06 (0.05)	0.48 (0.4)	1.95 (1.6)	>31.3 (>25.8)	>31.3 (>26)	>31.3 (>25.8)
FLC	62.5 (204.1)	>125 (>408.1)	15.6 (50.9)	>125 (>408.1)	>125 (>408.1)	62.5 (204.1)	62.5 (204.1)	>31.3 (>102.2)	>31.3 (>102.2)	1.95 (6.4)	62.5 (204.1)	62.5 (204.1)	62.5 (204.1)
ITC	0.5 (0.71)	>62.5 (>88.6)	7.8 (11.1)	31.3 (44.4)	31.3 (44.4)	31.3 (44.4)	31.3 (44.4)	7.8 (11.1)	0.48 (0.68)	0.12 (0.17)	0.48 (0.68)	0.195 (0.28)	0.975 (1.4)
POS	0.5 (0.71)	>62.5 (>89.2)	7.8 (11.1)	31.3 (44.7)	31.3 (44.7)	15.6 (22.3)	15.6 (22.3)	0.12 (0.17)	0.06 (0.09)	<0.03 (<0.04)	0.24 (0.34)	0.195 (0.28)	0.48 (0.68)
VOR	0.24 (0.69)	3.9 (11.2)	1.95 (5.6)	1.95 (5.6)	0.975 (2.8)	7.8 (22.3)	1.95 (5.6)	0.06 (0.17)	0.12 (0.34)	<0.03 (<0.06)	0.24 (0.69)	0.03 (0.06)	0.12 (0.34)

Yeast strains: A = *Candida albicans* ATCC 10231, **B** = *C. albicans* ATCC 64124, **C** = *C. albicans* ATCC MYA-2876(S), **D** = *C. albicans* ATCC 90819(R), **E** = *C. albicans* ATCC MYA-2310(S), **F** = *C. albicans* ATCC MYA-1237(R), **G** = *C. albicans* ATCC MYA-1003(R), **H** = *Candida glabrata* ATCC 2001, **I** = *Candida krusei* ATCC 6258, **J** = *Candida parapsilosis* ATCC 22019. NOTE: Here, the (S) and (R) indicate that ATCC reports these strains to be susceptible (S) and resistant (R) to ITC and FLC.
Filamentous fungi: K = *Aspergillus flavus* ATCC MYA-3631, **L** = *Aspergillus nidulans* ATCC 38163, **M** = *Aspergillus terreus* ATCC MYA- 3633.
Known antifungal agents: AmB = amphotericin B, CAS = caspofungin, FLC = fluconazole, ITC = itraconazole, POS = posaconazole, and VOR = voriconazole.
[a] For yeast strain **A, I, J**, and all filamentous fungi MIC-0 are reported for compounds **1**–**9** and **24**–**41**, whereas for all other strains tested, MIC-2 are reported for these compounds. *Note*: MIC-0 values are reported for AmB and CAS, whereas MIC-2 values are reported for the control azoles FLC, ITC, POS, and VOR.

excellent antifungal activity (<0.03–0.06 μg/mL) against *C. krusei* ATCC 6258 (strain **I**) and *C. parapsilosis* ATCC 22019 (strain **J**). When looking at compounds **5**–**7** with alkyl chains of medium lengths, we observed that they exerted moderate to excellent antifungal activity against all the strains tested with the exception of *A. flavus* ATCC MYA-3631 (strain **K**) against which they displayed poor activity. We noticed that compound **7** exerted the best antifungal activity followed by compound **6** and then by compound **5**. In general, compound **7** showed excellent antifungal activity against 10 (strains **A**, **C-J**, and **L**), moderate antifungal activity against two (strains **B** and **M**), and poor activity against *A. flavus* ATCC MYA-3631 (strain **K**) out of the 13 fungal strains tested. Similarly, compound **6** showed excellent antifungal activity against 9 (strains **A**, **C-G**, **I-J**, and **L**), and moderate antifungal activity against 4 (strains **B**, **E**, **H**, and **M**) out of the 13 fungal strains tested. Finally, compound **5** exhibited excellent antifungal activity against 5 (strains **A**, **G**, **I**, **J**, and **L**), moderate antifungal activity against 4 *C. albicans* strains (**B**–**F**) and *C. glabrata* ATCC 2001 (strain **H**), and poor antifungal activity against two *Aspergillus* strains (**K** and **M**) of the 13 strains tested. From these observations, we determined that increasing the length of the alkyl chain substituent results in an overall increase in antifungal activity. This prompted us to explore compounds **8** and **9** with longer C_{10} and C_{12} alkyl moieties. We observed that compounds **8** and **9** displayed poor antifungal activity against 5 (strains **B**, **D**, **F**, **G**, and **K**) and 6 (strains **A**, **B**, **D**, **G**, **I**, and **K**) out of 13 fungal strains, respectively. Additionally, we observed moderate inhibition of 5 (strains **A**, **C**, **E**, **H**, and **M**) and 4 (strains **C**, **E**, **F**, and **M**) of the 13 strains tested by compounds **8** and **9**, respectively. On the basis of our in-depth analysis of the MIC data for compounds **1**–**9**, we concluded that the optimal chain length required for the FLC derivatives to confer maximal antifungal activity were C_5-C_8.

(ii) Are these optimal C_5-C_8 chain lengths for our FLC derivatives the same as that of other families of *n*-alkylated molecules (*e.g.*, aminoglycoside, benzimidazole, and ebsulfur derivatives)? In recent years, the addition of linear alkyl chains to drug scaffolds has gained popularity as it has been demonstrated that it improves the activity of the compounds when compared to the parent non-alkylated molecules. Aminoglycosides are of the families of antibiotics to which alkyl chains have been added. It was shown that the optimal chain lengths required for maximal antifungal activity were C_8 for kanamycin A [17] and C_{12}-C_{14} for kanamycin B [4] and tobramycin [5]. For *n*-alkylated ebsulfur derivatives, the optimal chain lengths required to confer antifungal activity were reported to be C_5-C_6 [6]. Interestingly, alkyl chains varying in length between 1 and 3 carbons (C_1-C_3) were found to confer optimal antifungal activity when attached to a benzimidazole core [16]. From these data, it is clear that there is no universal alkyl chain length that can be utilized to confer prime antifungal activity. Consequently, one should always test a range of alkyl chains to determine the optimal length(s) for any new drug scaffold to be derivatized.

(iii) For a given alkyl chain length, would a 2,4-dichlorinated phenyl ring confer better or worse antifungal activity than the 2,4-difluorinated phenyl ring? Although compound **7** was identified as the best antifungal in our series based on its MIC values, we were concerned that the longer alkyl chain (C_8) could cause potential toxicity to mammalian cells. Therefore, for the next step in our SAR study, we decided to keep the length of the alkyl chains present in compounds **24**–**29** between 1 and 6 carbons. To explore the importance of the two fluoro groups on the phenyl ring of compounds **1**–**6**, we generated their dichlorinated counterparts **24**–**29**. By performing a pairwise comparison of for the MIC values of compounds **1** *versus* **24**, **2** *versus* **25**, **3** *versus* **26**, **4** *versus* **27**, **5** *versus* **28**, and **6** *versus* **29**, we concluded that the compounds with the 2,4-dichlorinated phenyl ring always displayed better antifungal activity than those containing the two fluoro groups. We also determined that the compounds displaying the best overall antifungal activity in this series were **28** and **29**, which contained C_5 and C_6 alkyl chains, respectively. For this reason, we next generated molecules **30**–**41** with C_5 and C_6 alkyl chains and mono-substituted (with fluoro or chloro) phenyl rings.

(iv) For a mono-substituted phenyl ring, which halogen substituent (fluoro or chloro) is best? After establishing that two chloro groups were better than two fluoro moieties, we wanted to determine if the trend would remain when only one halogen substituent was attached to the phenyl ring. In order to shed light on this, we synthesized 12 additional compounds, **30**–**41**, and performed direct pairwise comparisons (**30** *versus* **36**, **31** *versus* **37**, **32** *versus* **38**, **33** *versus* **39**, **34** *versus* **40**, **35** *versus* **41**) in terms of their activity against the fungal strains tested based on our MIC data (Table 1). Interestingly, in contrary to what we observed with the dihalogenated molecules, we found that, in most cases, the mono-substituted phenyl rings with a fluoro substituent displayed better antifungal activity than its counterpart with a chloro substituent. Briefly, we found that compound **30** (with a 2-F) repeatedly showed better antifungal activity against *C. albicans* strains, non-*albicans Candida* strains, and *Aspergillus* strains when compared to its counterpart **36** (with a 2-Cl). Compounds **31** (with a 2-F) and **32** (with a 3-F) displayed better antifungal activity against *C. albicans* as well as non-*albicans Candida* strains, whereas their respective counterpart **37** and **38** showed better activity only against *Aspergillus* strains. Similarly, compound **33** (with a 3-F) exhibited better antifungal activity against *C. albicans* strains than compound **39** (with a 3-Cl). Both compounds **33** and **39** showed almost identical activity against *Aspergillus* strains. Finally, compound **34** (with a 4-F) showed better antifungal activity against *C. albicans* strains, worse antifungal activity against non-*albicans Candida* strains, and equivalent antifungal activity against *Aspergillus* strains than its counterpart compound **40** (with a 4-Cl). Likewise, compound **35** (with a 4-F) showed worse antifungal activity against *C. albicans* as well as non-*albicans Candida* strains, but identical antifungal activity against *Aspergillus* strains than its counterpart compound **41** (with a 4-Cl).

(v) For a specific alkyl chain length, which level of substitution (mono- *versus* di-) confer the best antifungal activity? As previously mentioned, based on our MIC data we concluded that compounds with C_5-C_6 chain lengths confer the best antifungal activity. We next decided to explore the effect of the level of substitution (mono- *versus* di-) on the phenyl ring for our compounds with C_5-C_6 alkyl chain length based by comparing the MIC data obtained for compound **5** *versus* compounds **30**, **32**, and **34**; compound **6** *versus* compounds **31**, **33**, and **35**; compound **28** *versus* compounds **36**, **38**, and **40**; as well as compound **29** *versus* compounds **37**, **39**, and **41**. We noticed that the 2,4-difluorinated compounds **5** and **6** displayed inferior antifungal activity when compared to their mono-2-substituted counterparts **30** and **31**. On the other hand, both compounds **5** and **6** showed better antifungal activity than their mono-3-substituted counterparts **32** and **33**, and than their mono-4-substituted counterparts **34** and **35**. Interestingly, we observed that the 2,4-dichlorinated compounds **28** and **29** always displayed better antifungal activity than any of their mono-substituted counterparts.

(vi) For a given substituent (fluoro of chloro), what is the optimal position (*ortho*, *meta*, or *para*) for mono-substitution on the phenyl ring? Finally based on a direct comparison of compounds **30** *versus* **32** *versus* **34**; compounds **31** *versus* **33** *versus* **35**; compounds **36** *versus* **38** *versus* **40**; compounds **37** *versus* **39** *versus* **41**, we found that substitution at the *ortho* position (compounds **30**, **31**, **36**, and **37**) confers greater antifungal activity than does that at the *para* position (compounds **34**, **35**, **40**, and **41**). Substitution at the *meta* position (compounds **32**, **33**, **38**, and **39**) is the least optimal to

confer antifungal activity.

2.4. Time-kill assay

Our in-depth SAR analysis has identified several potential newly synthesized antifungals among which compounds **6** and **29** stood to be the best. Therefore, compounds **6** and **29** were further examined for their antifungal potency against a representative *C. albicans* strain ATCC 10231 (strain **A**) by performing a time-kill assay over a 24-h period (Fig. 3). In general, we observed dose-dependent killing effect by our compounds **6** and **29** as well as by a reference drug voriconazole (VOR) against *C. albicans* ATCC 10231 (strain **A**). At 0.5× MIC, compounds **6** and **29** showed fungistatic effects against strain **A** for the first 12 h of growth, and after that the growth was increased by 1 $\log_{10}$ CFU at 24 h for both of these compounds. Similarly, at 1× MIC, both compounds maintained the fungistatic effect up until 12 h followed by a reduction in CFU of strain **A** by 1 $\log_{10}$ by compound **6** and 0.25 $\log_{10}$ by compound **29** at 24 h. However, at 4× MIC, compounds **6** and **29** displayed fungicidal effect by achieving ≥2 $\log_{10}$ and 1.25 $\log_{10}$ reduction of CFU of strain **A** at 9 h and 6 h, respectively (Fig. 3). More importantly, we also noticed that our compounds **6** and **29** showed either superior or comparable growth inhibitory effect against *C. albicans* ATCC 10231 (strain **A**) when compared to the reference drug (VOR) under similar conditions. No antifungal carryover effect was observed with compounds **6** and **29** against tested strain **A** at 0.5×, 1×, or 4×MICs.

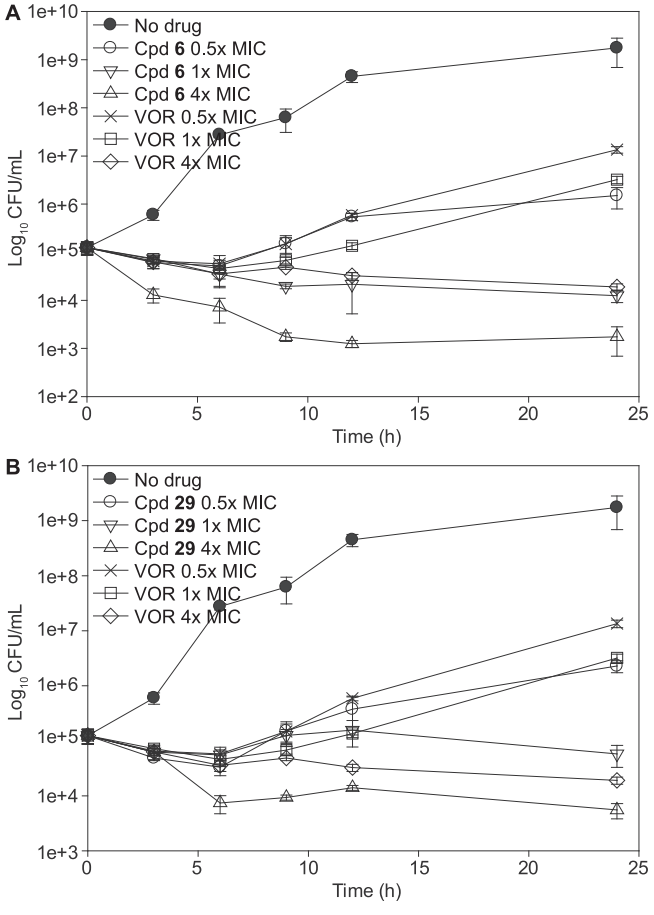


Fig. 3. Representative time-kill studies of compounds **6** and **29**, and VOR against *C. albicans* ATCC 10231 (strain **A**). Yeast cells were either treated with **A.** compound **6** (white circle (0.5× MIC), inverted white triangle (1× MIC), and white triangle (4× MIC)) or **B.** compound **29** (white circle (0.5× MIC), inverted white triangle (1× MIC), and white triangle **A** and **B** VOR was used as a control (cross (0.5× MIC), white square (1× MIC), and white diamond (4× MIC)). A no drug control (black circle) is also presented in both panels.

2.5. Hemolysis assay

With the promising antifungal activity of our newly synthesized alkylated FLC derivatives, we wanted to confirm that they would display none or reasonable (*i.e.*, <50% at 10× MIC) hemolytic activity against red blood cells. In order to determine the selectivity of our FLC derivatives towards fungal cells, we selected our 13 best compounds (**4**–**7**, **27**–**31**, **35**–**37**, and **41**) out of 27 to evaluate their hemolytic effect against mouse red blood cells (mRBCs) (Fig. 4, Fig. S73, and Table S1). As observed with their antifungal activity, compounds **4**–**7** also showed chain-length-dependent hemolytic activity on mRBCs in the order of **4** (C_4) < **5** (C_5) < **6** (C_6) < **7** (C_8). Briefly, no measurable hemolysis was detected for compound **4** even at the highest concentration of 125 μg/mL used. Likewise, compounds **5** and **6** lysed 45% of mRBCs at 125 μg/mL (4–4096-folds higher concentration than their antifungal MIC values) and 26% of mRBCs at 62.5 μg/mL (4–2048-folds higher concentration than their antifungal MIC values), suggesting a minimal hemolytic effect by compounds **5** and **6**. On the other hand, compound **7** induced 47% lysis of mRBCs at 7.8 μg/mL, which was either lower concentration or 64-fold higher concentration than its antifungal MIC values. Although compound **7** with a C_8 alkyl chain was one of the most active compound, it readily lysed the mRBCs as well and, therefore, we were right about our hypothesis that the longer chain could be problematic. Next, we analyzed the hemolytic activity of the dichlorinated compounds **27**–**29** and compared them with that

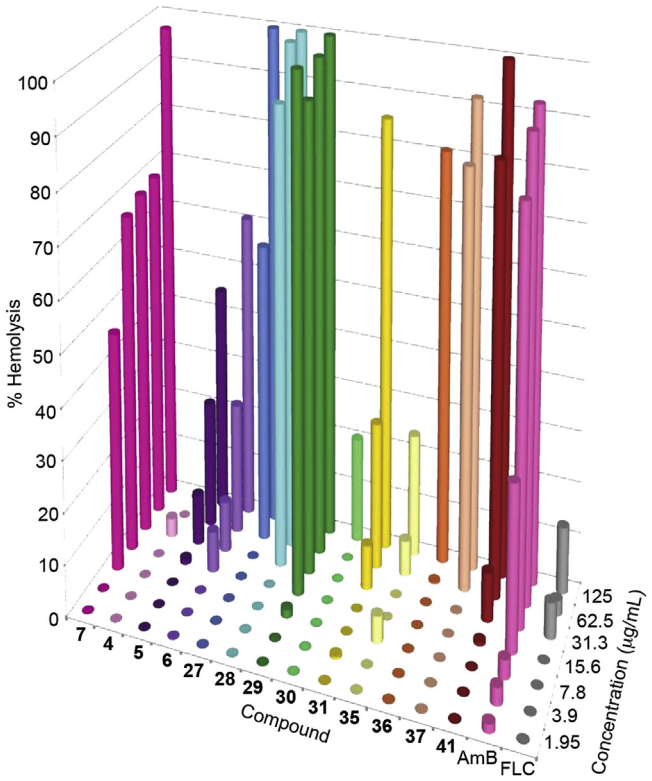


Fig. 4. 3D bar graph depicting the dose-dependent hemolytic activity of FLC derivatives against mouse erythrocytes. Mouse erythrocytes were treated and incubated for 1 h at 37 °C with compounds **4**–**6**, **27**–**31**, **35**–**37**, **41**, AmB, and FLC at concentrations ranging from 1.95 to 125 μg/mL. Triton X-100® (1% *v*/*v*) was used as a positive control (100% hemolysis, not shown).

of their difluorinated counterparts **4**–**6**. Like compounds **4**–**6**, compounds **27**–**29** showed the chain-length-dependent hemolytic activity in the order of **27** (C_4) < **28** (C_5) < **29** (C_6). However, the 2,4-dichlorinated compounds were more hemolytic against mRBCs when compared to their 2,4-difluorinated counterparts. Concisely, compound **27** lysed 59% of mRBCs at 62.5 μg/mL, which was 16 to 2048-folds higher concentration than its antifungal MIC values. Similarly, compound **28** showed no detectable hemolysis at up to 15.6 μg/mL (4–512-folds higher concentration than its antifungal MIC values), but significantly increased by 90% at above that concentration. Additionally, compound **29** did not show hemolysis at up to 7.8 μg/mL, but the effect was bumped up to 100% at the concentration above that. We also found that the 2,4-difluorinated compounds **5** and **6** displayed either more or equal hemolytic activity than their mono-fluorinated counterparts **30** and **31**. However, the 2,4-difluorinated compounds **5** and **6** were always less hemolytic than their mono-chlorinated counterparts **36** and **37**. On the other hand, the dichlorinated compounds **28** and **29** consistently showed more hemolytic activity than their mono-fluorinated (**36** and **37**) as well as mono-chlorinated (**30** and **31**) counterparts against mRBCs. Finally, by performing pairwise comparisons of the hemolytic activity of our compounds (**30** *versus* **36**, **31** *versus* **37**, and **35** *versus* **41**), we evaluated the effects of the halide substituent identity (fluoro *versus* chloro) and position(s) (*ortho versus para*) on the phenyl ring. In general, the 2-F group (compounds **30** and **31**) resulted in less hemolytic effect than the 2-Cl (compounds **36** and **37**). Likewise, the 4-F (compound **35**) exhibited less hemolytic activity than the 4-Cl (compound **41**). Notably, we noticed that the majority of our newly synthesized FLC derivatives displayed less hemolytic effect than the control drug AmB.

2.6. Cytotoxicity assay

Fungi are eukaryotes that share similar cellular and biochemical features with mammalian cells. As a result, the drugs that are designed to target fungi could cause unwanted side effects on mammalian cells. To further determine the selectivity of our FLC derivatives towards fungal cells, we tested our 16 best compounds (**4**–**7**, **26**–**31**, **34**–**37**, and **40**–**41**) for their toxicity against two nucleated mammalian cell lines, A549 and BEAS-2B (Fig. S74). The EC_{50} values from the bar graphs presented in Fig. S74 are also summarized in Table 2. In parallel, we also used two FDA approved antifungal agents, POS and FLC, as comparators. Encouragingly, we observed that the majority of our difluorinated compounds (**4**–**6**, with the exception of **7**) were generally non-toxic to both mammalian cell lines tested with EC_{50} values of >31.3 μg/mL. These EC_{50} values are 4 to 1024-folds higher than the respective antifungal MIC values for these derivatives. Although compound **7** (one of the best antifungal in the series) was relatively toxic among the difluorinated group, it showed no toxicity at up to a concentration of 7.8 μg/mL against both mammalian cell lines. Similarly, our dichlorinated compounds **26**–**29** were also found to be non-toxic as they allowed for >85% cell survival at 15.6 μg/mL. Additionally, no cytotoxicity was detected for our compounds with 2-F (compounds **30** and **31**), 4-F (compounds **34** and **35**), 2-Cl (compounds **36** and **37**), and 4-Cl (compounds **40** and **41**) against both cell lines tested, with EC_{50} values of >31.3 μg/mL. After determining the EC_{50} values of our compounds (**4**–**7**, **26**–**31**, **34**–**37**, and **40**–**41**) against mammalian cell lines, we calculated the cell selectivity index (SI) values of these compounds against all fungal strains (Table S2). The SI for a compound is defined as the ratio of its EC_{50} value against a mammalian cell line (*e.g.*, A549 or BEAS-2B) to its MIC value against a specific fungal strain. Here, we considered SI values ≥ 8 to be good in terms of selectivity against fungal cells. All of our compounds (**4**–**7**, **26**–**31**, **34**–**37**, and **40**–**41**) were highly selective against the majority of the non-*albicans Candida* strains with SI values ranging from 16 to 1167. Interestingly, our best compounds **26**–**29** showed higher selectivity against most of *C. albicans* strains with SI values ranging from 8 to 138 as well as against *Aspergillus* strains with SI values ranging from 8 to 130. Interestingly, our FLC derivatives exhibited better safety profile than the FDA approved drug AmB (only 30% cell survival at 15.6 μg/mL).

Table 2
Cytotoxicity (EC_{50}, μg/mL) of selected novel FLC analogues against A549 and BEAS-2B cell lines. Values are presented as mean ± SDEV.

Cpd#	A549	BEAS-2B
4	>31.3	36 ± 4
5	>31.3	>31.3
6	35 ± 5	>31.3
7	12 ± 2	13 ± 3
26	>31.3	>31.3
27	>31.3	>31.3
28	33 ± 2	>31.3
29	20 ± 8	28 ± 1
30	37 ± 6	>31.3
31	>31.3	>31.3
34	>31.3	33 ± 3
35	>31.3	>31.3
36	>31.3	>31.3
37	>31.3	>31.3
40	>31.3	>31.3
41	38 ± 4	>31.3
POS	37 ± 3	26 ± 3
FLC	>62.5	>62.5

2.7. Membrane permeabilization assay

We previously demonstrated that amphiphilic molecules can exert their activity by disrupting the membrane of fungal cells [5,6,16–19]. Based on these findings, we also speculated that our alkylated FLC derivatives could potentially cause antifungal activity by disrupting the fungal membrane. To investigate the mechanism of action of our FLC derivatives, we tested compounds **5**–**8** and **29** to evaluate their effect on fungal membrane integrity using propidium iodide dye (PI) (Fig. 5). PI is a membrane impermeable dye that cannot enter the intact cell unless the cell membrane is damaged. Using these compounds, we also wanted to determine how do the lengths of the alkyl side chains correlate with the membrane disruption potential of these antifungal agents. We also used a kanamycin B derivative, KANB (C_{14}) with a linear alkyl chain of 14 carbons, and FLC as positive and negative controls, respectively. We have previously demonstrated that KANB (C_{14}) at its MIC (7.8 μg/mL) significantly increases the PI dye uptake in *C. albicans* ATCC 64124 (strain **B**). Interestingly, we found that none of our compounds **5**–**8** and **29** (at either 1× MIC or 4× MIC) induced cellular uptake of PI dye into *C. albicans* ATCC 10231 (strain **A**) regardless of their alkyl chain length. These data indicated that the antifungal mode of action of our FLC derivatives is not membrane disruption.

2.8. Determination of sterol composition

Based on our membrane permeabilization assay, we ascertained that none of our FLC derivatives are able to cause membrane disruption of *C. albicans* ATCC 10231 (strain **A**) (Fig. 5). This prompted us to investigate by gas chromatography-mass spectrometry (GC-MS) the potential of our FLC derivatives to exert their antifungal action by inhibiting the sterol 14α-demethylase enzyme of the ergosterol biosynthetic pathway, similarly to the parent FLC (Fig. 6A). We selected one of our best compound, **29**, to evaluate its

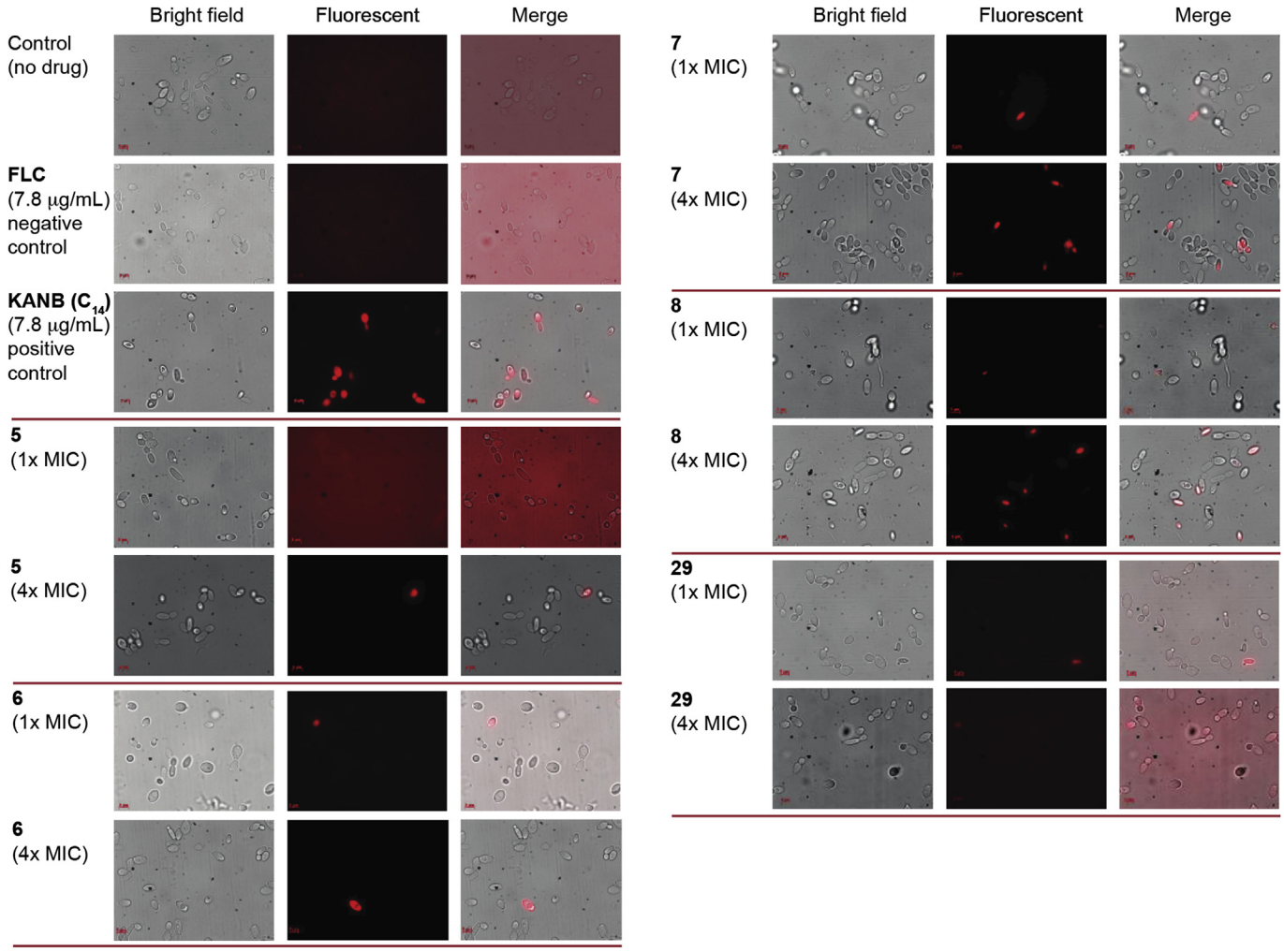
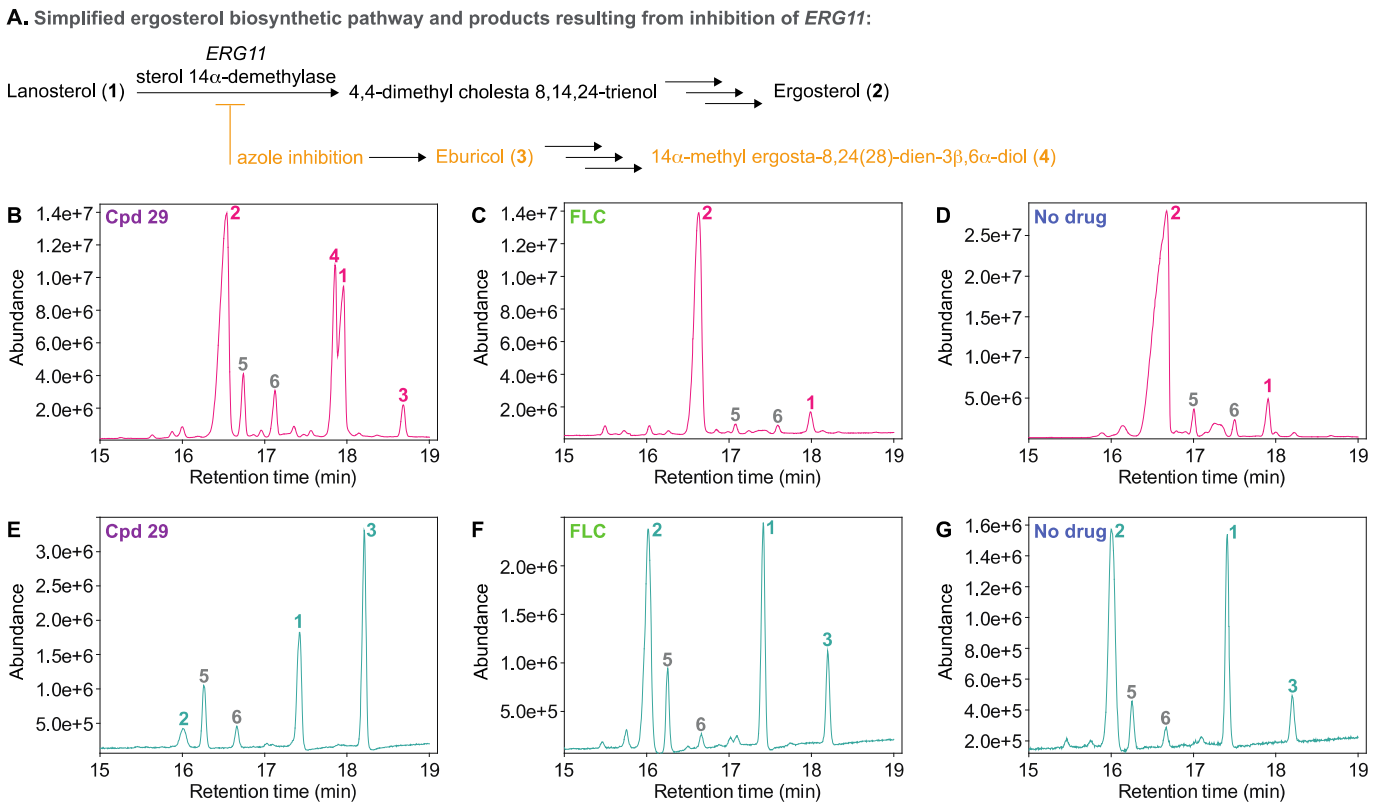


Fig. 5. Effect of FLC and its analogues **6** and **29** on the cell membrane integrity of *C. albicans* ATCC 10231 (strain **A**). From top to bottom: propidium iodide (PI) dye uptake by yeast cells without drug, with FLC (7.8 μg/mL), KANB (C_{14}) (7.8 μg/mL), compound **6** (at 1× MIC and 4× MIC), and compound **29** (at 1× MIC and 4× MIC).

effect on sterol composition of two strains, *C. albicans* ATCC 10231 (strain **A**, Fig. 6B) and *C. albicans* ATCC 64124 (strain **B**, Fig. 6E), at the sub-MIC levels of 0.125 μg/mL and 1.95 μg/mL, respectively. We also used FLC at 0.125 μg/mL (against strain **A**, Fig. 6C) and at 1.95 μg/mL (against strain **B**, Fig. 6F) as a comparator. No drug controls were also performed (Fig. 6D and G). Based on our sterol profile results (summarized in Fig. 6H), we determined that in the absence of an azole drug strain **A** accumulated ~91% ergosterol, suggesting that sterol biosynthesis was fully functional in this fungal strain. Interestingly, in the absence of an azole drug we observed a different trend against strain **B**. Indeed, strain **B** accumulated a relatively low amount of ergosterol (~54%) and an increased amount of lanosterol (~29%) and eburicol (~6%) in comparison to what was observed with strain **A**. It is important to note that *C. albicans* ATCC 64124 (strain **B**) is an azole-resistant strain with a mutation in the sterol 14α-demethylase enzyme, which probably contributed to its different sterol profile. Similarly, when strain **A** was treated with FLC (0.125 μg/mL), ergosterol (~93%) was found to be the predominant sterol in the cells, indicating that FLC had no effect on ergosterol biosynthesis in this specific fungal strain. This observation could be easily explained by the fact that the concentration of FLC utilized in the assay was ~500-fold lower than the antifungal MIC value (62.5 μg/mL) for FLC against this strain. However, when strain **B** was treated with FLC (1.95 μg/mL), we detected a relatively low amount of ergosterol (~48%) and an increased amount of lanosterol (~30%) and eburicol (~12%) in comparison to what was observed with strain **A**. In contrary to what we observed with the non-treated and FLC-treated strain **A**, with compound **29**, we detected a reduction in the amount of ergosterol (~49%) with the concomitant increase in the amount of lanosterol (~19%), eburicol (~3%), and the fungistatic metabolite 14α-methylergosta-8,24,(28)-dien-3β,6α-diol (~19%). Interestingly, when strain **B** was treated with compound **29**, we saw a significant decrease in the amount of ergosterol (~6%) and a drastic increase in the amount of eburicol (~48%). In addition, no traces of the fungistatic metabolite 14α-methylergosta-8,24,(28)-dien-3β,6α-diol were detected in compound **29**-treated strain **B**. These results indicated that our compound **29** inhibits the sterol 14α-demethylase enzyme of fungal cells, and thereby affects the ergosterol biosynthetic pathway.

3. Conclusion

In summary, by developing a series of 27 new alkylated-FLC derivatives, we identified numerous promising antifungal agents with low hemolytic activity, low cytotoxicity, and great activity against *C. albicans*, non-*albicans Candida*, and *Aspergillus* strains. We showed that in contrary to what has generally been observed as a



H. Sterol composition of untreated and compound 29 or FLC-treated *C. albicans* strains:

	Sterol composition (%)					
	Cpd 29		FLC		No drug	
Sterol	**10231**	**64124**	**10231**	**64124**	**10231**	**64124**
Lanosterol (**1**)	19.30	28.99	3.81	30.59	4.17	29.38
Ergosterol (**2**)	49.00	5.73	93.79	48.03	91.15	54.72
Eburicol (**3**)	2.93	47.97	ND	11.61	ND	6.28
14α-methyl ergosta-8,24(28)-dien-3β,6α-diol (**4**)	18.81	ND	ND	ND	ND	ND
Unknown sterol (**5**)	5.57	13.04	1.12	9.75	2.78	6.98
Unknown sterol (**6**)	4.36	4.25	1.25	0.02	1.88	2.67

ND = not detected

Fig. 6. A. A simplified ergosterol biosynthetic pathway and products resulting from inhibition of *ERG11*. **B-G.** GC-MS chromatograms of the sterols extracted from untreated and antifungals treated *C. albicans* strains. *C. albicans* ATCC 10231 (strain **A**) was treated with compound **29** (panel **B**) and FLC (panel **C**) at 0.125 μg/mL, or DMSO (no drug) as a control (panel **D**). Likewise, *C. albicans* ATCC 64124 (strain **B**) was treated with compound **29** (panel **E**) and FLC (panel **F**) at 1.95 μg/mL, or DMSO (no drug) as a control (panel **G**). The peaks are for lanosterol (**1**), ergosterol (**2**), eburicol (**3**), 14α-methyl ergosta 8,24(28)-dien-3β,6α-diol (**4**), and unknown sterols (**5** and **6**). **H.** A table summarizing the percentage of each sterol from panels **B-G**.

mechanism of action for molecules containing long alkyl chains (*i.e.*, membrane disruption), our novel alkylated-FLC derivatives do not disrupt the fungal membrane, but instead target the ergosterol biosynthetic pathway by inhibiting the sterol 14α-demethylase enzyme involved in the first committed step responsible for the conversion of lanosterol into 4,4-dimethyl cholesta 8,14,24-trienol. In the future, although out of scope for this study, it will be interesting to see how our new alkylated-FLC derivatives will fair in the translation process of moving towards clinical application. Work along these lines is currently underway in our laboratory.

Funding sources

This work was supported by startup funds from the College of Pharmacy at the University of Kentucky (to S.G.-T.).

Notes

The authors declare no competing financial interest.

Abbreviations

FLC, fluconazole; VOR, voriconazole; ITC, itraconazole; POS, posaconazole; AmB, amphotericin B; CAS, caspofungin; KANB, kanamycin B; TOB, tobramycin; PK, pharmacokinetic; ATCC, American Type Culture Collection; mRBCs, mouse red blood cells; PI, propidium iodide dye; SAR, structure-activity-relationship.

Acknowledgment

This work was supported by startup funds from the College of Pharmacy at the University of Kentucky (to S.G.-T.). We thank Dr. Joseph Chappell (University of Kentucky) for letting us use his gas

chromatography-mass spectrometer. We also thank Ms. Kristin Linscott (University of Kentucky) for training us on the fungal sterol extraction procedure and use of the GC-MS instrument. We also thank Drs. Lisa J. Vaillancourt and Jon S. Thorson (University of Kentucky), as well as Dr. Jon Y. Takemoto (Utah State University) for providing some of the fungal strains used in our study.

Appendix A. Supplementary data

Supporting Information. Experimental procedures for (i) the synthesis and characterization of compounds **1**–**9**, **24**–**41**, and all intermediates (ii) determination of MIC values against fungal strains, (iii) time-kill curves, (iv) hemolysis, (v) mammalian cell toxicity, (vi) membrane permeabilization assay using propidium iodide staining and (vii) determination of sterol composition in *C. albicans* pre- and post-treatment are included in the Supporting Information. The Supporting Information also includes supplementary Table S1, which provides the exact % of hemolysis associated with Fig. 4. A 2D representation of the data presented in Fig. 4 is also provided in Fig. S73. The raw data for cytotoxicity assays provided in Table 2 are presented in Fig. S74. A supplementary Table S2 is also included and provides SI values. ^{1}H and ^{13}C NMR spectra (Figs. S1-—S72) for the molecules generated are also provided. This material is available free of charge via the Internet.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2017.03.075.

References

[1] M.K. Kathiravan, A.B. Salake, A.S. Chothe, P.B. Dudhe, R.P. Watode, M.S. Mukta, S. Gadhwe, The biology and chemistry of antifungal agents: a review, Bioorg. Med. Chem. 20 (2012) 5678–5698.
[2] P.Z. Zhang, S.F. Zhou, T.R. Li, L. Jiang, Efficient synthesis and *in vitro* antifungal activity of 1H-benzimidazol-1-yl acetates/propionates containing 1H-1,2,4-triazole moiety, Chin. Chem. Lett. 23 (2012) 1381–1384.
[3] Z. Jiang, Y. Wang, W. Wang, S. Wang, B. Xu, G. Fan, G. Dong, Y. Liu, J. Yao, Z. Miao, W. Zhang, C. Sheng, Discovery of highly potent triazole antifungal derivatives by heterocycle-benzene bioisosteric replacement, Eur. J. Med. Chem. 64 (2013) 16–22.
[4] M.Y. Fosso, S.K. Shrestha, K.D. Green, S. Garneau-Tsodikova, Synthesis and bioactivities of kanamycin B-derived cationic amphiphiles, J. Med. Chem. 58 (2015) 9124–9132.
[5] S.K. Shrestha, M.Y. Fosso, K.D. Green, S. Garneau-Tsodikova, Amphiphilic tobramycin analogues as antibacterial and antifungal agents, Antimicrob. Agents Chemother. 59 (2015) 4861–4869.
[6] H.X. Ngo, S.K. Shrestha, S. Garneau-Tsodikova, Identification of ebsulfur analogues with broad-spectrum antifungal activity, ChemMedChem 11 (2016) 1507–1516.
[7] H.X. Ngo, S.K. Shrestha, K.D. Green, S. Garneau-Tsodikova, Development of ebsulfur analogues as potent antibacterials against methicillin-resistant *Staphylococcus aureus*, Bioorg. Med. Chem. 24 (2016) 6298–6306.
[8] J. Loeffler, D.A. Stevens, Antifungal drug resistance, Clin. Infect. Dis. 36 (2003) S31–S41.
[9] A.M. Bal, The echinocandins: three useful choices or three too many? Int. J. Antimicrob. Agents 35 (2010) 13–18.
[10] J.C.R. Correa, H.R.N. Salgado, Review of fluconazole properties and analytical methods for its determination, Crit. Rev. Anal. Chem. 41 (2011) 124–132.
[11] P. Kale, L.B. Johnson, Second-generation azole antifungal agents, Drugs Today 41 (2005) 91–105.
[12] C. Girmenia, E. Finolezzi, New-generation triazole antifungal drugs: review of the Phase II and III trials, Clin. Invest. 1 (2011) 1577–1594.
[13] C. Ruden, S.O. Hansson, How accurate are the European Union's classifications of chemical substances, Toxicol. Lett. 144 (2003) 159–172.
[14] D. Chopra, T.P. Mohan, K.S. Rao, T.N.G. Row, 2-(2,4-Dichlorophenyl)-1-(1H-1,2,4-triazol-1-yl)hexan-2-ol (hexaconazole), Acta Crystallogr. E 60 (2004) 2410–2412.
[15] Y. Ueda, J.D. Matiskella, J. Golik, T.P. Connolly, T.W. Hudyma, S. Venkatesh, M. Dali, S.H. Kang, N. Barbour, R. Tejwani, S. Varia, J. Knipe, M. Zheng, M. Mathew, K. Mosure, J. Clark, L. Lamb, I. Medin, Q. Gao, S. Huang, C.P. Chen, J.J. Bronson, Phosphonooxymethyl prodrugs of the broad spectrum antifungal azole, ravuconazole: synthesis and biological properties, Bioorg. Med. Chem. Lett. 13 (2003) 3669–3672.
[16] N. Thamban Chandrika, S.K. Shrestha, H.X. Ngo, S. Garneau-Tsodikova, Synthesis and investigation of novel benzimidazole derivatives as antifungal agents, Bioorg. Med. Chem. 24 (2016) 3680–3686.
[17] C.-W. Chang, M. Fosso, Y. Kawasaki, S. Shrestha, M.F. Bensaci, J. Wang, C.K. Evans, J.Y. Takemoto, Antibacterial and antifungal conversion of neamine aminoglycosids through alkyl modification. Strategy for reviving old drugs into agrofungicides, J. Antibiot. 63 (2010) 667–672.
[18] S.K. Shrestha, M.Y. Fosso, S. Garneau-Tsodikova, A combination approach to treating fungal infections, Sci. Rep. 5 (2015) 17070.
[19] S.K. Shrestha, C.W. Chang, N. Meissner, J. Oblad, J.P. Shrestha, K.N. Sorensen, M.M. Grilley, J.Y. Takemoto, Antifungal amphiphilic aminoglycoside K20: bioactivities and mechanism of action, Front. Microbiol. 5 (2014) 671.