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Research paper

Novel alkylated azoles as potent antifungals

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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}

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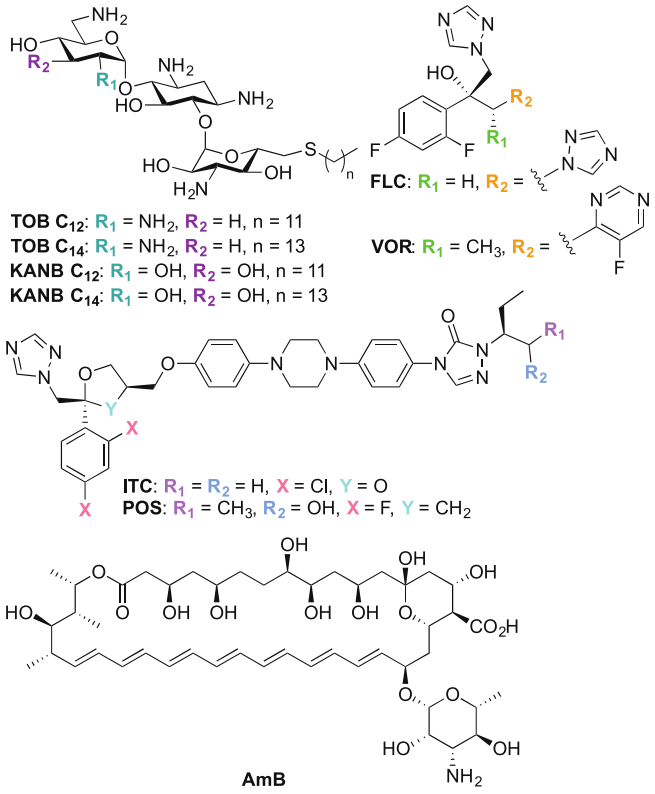

Fig. 1. Structures of compounds discussed in this study.

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