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Synthesis and evaluation of thiophene-based guanylhydrazones (iminoguanidines) efficient against panel of voriconazole-resistant fungal isolates

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ABSTRACT

A series of new thiophene-based guanylhydrazones (iminoguanidines) were synthesized in high yields using a straightforward two-step procedure. The antifungal activity of compounds was evaluated against a wide range of medicaly important fungal strains including yeasts, molds, and dermatophytes in comparison to clinically used drug voriconazole. Cytotoxic properties of compounds were also determined using human lung fibroblast cell line and hemolysis assay. All guanylhydrazones showed significant activity against broad spectrum of clinically important species of *Candida* spp., *Aspergillus fumigatus*, *Fusarium oxysporum*, *Microsporum canis* and *Trichophyton mentagrophytes*, which was in some cases comparable or better than activity of voriconazole. More importantly, compounds **10**, **11**, **13**, **14**, **18** and **21** exhibited excellent activity against voriconazole-resistant *Candida albicans* CA5 with very low minimal inhibitory concentration (MIC) values <2 μg mL^{-1}. Derivative **14**, bearing bromine on the phenyl ring, was the most effective compound with MICs ranging from 0.25 to 6.25 μg mL^{-1}. However, bis-guanylhydrazone **18** showed better selectivity in terms of therapeutic index values. In vivo embryotoxicity on zebrafish (*Danio rerio*) showed improved toxicity profile of **11**, **14** and **18** in comparison to that of voriconazole. Most guanylhydrazones also inhibited *C. albicans* yeast to hyphal transition, essential for its biofilm formation, while **11** and **18** were able to disperse preformed *Candida* biofilms. All guanylhydrazones showed the equal potential to interact with genomic DNA of *C. albicans* in vitro, thus indicating a possible mechanism of their action, as well as possible mechanism of observed cytotoxic effects. Tested compounds did not have significant hemolytic effect and caused low liposome leakage, which excluded the cell membrane as a primary target. On the basis of computational docking experiments using both human and cytochrome P450 from *Candida* it was concluded that the most active guanylhydrazones had minimal structural prerequisites to interact with the cytochrome P450 14α-demethylase (CYP51). Promising guanylhydrazone derivatives also showed satisfactory pharmacokinetic profile based on molecular calculations.

1. Introduction

Fungal infections have received less attention over past decades, however mortality and morbidity rate of opportunistic fungal infections is exponentially increasing and the number of fatal incidence due to fungi is becoming comparable with that of tuberculosis and malaria.[1] Pathogenic fungi, particularly *Candida* and *Aspergillus* species are one of the most common diagnosed opportunistic infections and are the causes of significant mortality in immunocompromised patients.[2] On the other side, less threatening fungal skin infections were in the top 10 most prevalent diseases worldwide in 2010, affecting 984 million people[3] and posing enormous health burden globally. Given that the majority of infections caused by *Candida albicans* are biofilm-related, and that this form is

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usually more resistant to traditional antifungal treatment it adds to recurrence and chronicity of the disease.[4,5]

Antifungal agents are mainly divided into four classes: polyenes, pyrimidines, echinocandins and azoles (Fig. 1).[6] The polyene amphotericin B was developed in 1953, and by far is the most potent antifungal agent for almost all invasive fungal infections. It binds to ergosterol, which is the major sterol in fungal cell membranes, and thereby forming membrane-spanning channels that lead to the leakage of intracellular constituents and finally fungal cell death. The most frequent side effects associated with amphotericin B are infusional toxicity and nephrotoxicity. Flucytosine, pyrimidine-base antifungal was synthesized in 1957 as a potential antitumor drug but later on it was discovered that it had significant fungistatic activity. Flucytosine itself has no antifungal activity but it is converted to 5-fluorouracil within fungal cell, which then inhibits DNA and RNA synthesis. Usually, it is used in combination therapy with amphotericin B. The echinocandin derivative caspofungin inhibits the synthesis of β-(1,3)-D-glucan, an important component of the fungal cell wall. The major disadvantages associated with caspofungin are narrow spectrum of activity (only active against *Candida* spp. and *Aspergillus* spp.) and poor oral bioavailability. Voriconazole is a member of clinically important triazoles that are widely used for serious invasive fungal infections, as well as for dermal mycoses.[7] Voriconazole has fungistatic activity against *Candida* spp. and *Fusarium* spp. and with fungicidal activity against *Aspergillus* spp. The mechanism of action of voriconazole is inhibition of fungal cytochrome P450-mediated (14α-lanosterol demethylation, an essential step in the synthesis of ergosterol. This eventually causes the depletion of ergosterol and disrupts the integrity of fungal cell membranes, eventually leading to cell lysis.[8,9] The most common side effects of voriconazole therapy include visual disturbances, skin rashes, elevation of hepatic enzyme levels, headache, and hallucinations.[10–12]

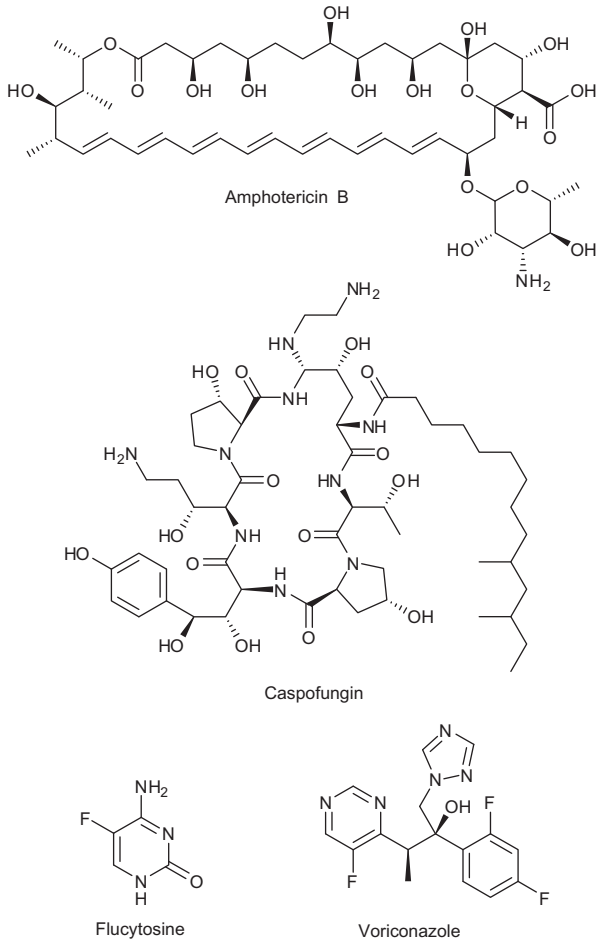

Figure 1. Clinically used antifungals.

The increasing number of reports of fungal infections among immunocompromised patients and additional problems associated with toxicity and resistance to standard antifungal drugs cause an urgent need for the development of novel safe and effective antifungals.[13] Apart from lower toxicity and higher selectivity, panfungal (broad spectrum) activity is also very desirable property that novel antifungals should have.

Compounds containing imonoguanidine moiety have been known for a while and explored for their wide variety of biological activities. Antiviral,[14–16] antiparasitic,[17–19] and antibacterial[20–23] activities of this versatile group of compounds have been described. However, their antifungal potential has not been extensively explored.[24] More importantly, to the best of our knowledge, they have not been evaluated against human fungal pathogens.

In a view of the general lack of novel antifungal agents and in a continuation of our studies toward the discovery of biologically active heterocyclic molecules, herein we report the synthesis and structural characterization of novel guanylhydrazones with their subsequent in vitro biological evaluation for antifungal activity including activity against fungal biofilms, their cytotoxic properties, in vitro DNA interaction ability and in silico analysis. In vivo embryotoxicity on zebrafish (*Danio rerio*) of the most promising derivatives has been determined. The druglikeness of studied compounds was also established using Lipinski's 'rule of five'.[25,26]

2. Materials and methods

2.1. Instrumentation

Melting points were determined on a Boetius PMHK apparatus and were not corrected. IR spectra were recorded on a Thermo-Scientific Nicolet 6700 FT-IR Diamond Crystal. NMR: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Ultrashield Advance III spectrometer (at 500 and 125 MHz, respectively) using tetramethylsilane (TMS) as the internal standard. The NMR solvents are specified individually for each compound. Chemical shifts are expressed in parts per million (ppm) on the (δ) scale. Chemical shifts were calibrated relative to those of the solvents. ESI MS spectra of the synthesized compounds were recorded on an Agilent Technologies 6210 Time-of-Flight LC/MS instrument in positive ion mode using CH_3CN/H_2O = 1:11:1 with 0.2 % HCOOH as the carrying solvent solution. The samples were dissolved in pure acetonitrile (HPLC grade). The selected values were as follows: capillary voltage = 4 kV; gas temperature = 350 °C; drying gas = 12.l min^{-1}; nebulizer pressure = 45 psig; fragmentator voltage = 70 V. All yields reported refer to isolated yields. Compounds were analyzed for purity using: Agilent 1200 HPLC system equipped with Quat Pump (G1311B), Injector (G1329B) 1260 ALS, TCC 1260 (G1316A) and Detector 1260 DAD VL+(G1315C), and Waters 1525 HPLC dual pump system equipped with an Alltech Select degasser system, and a dual λ 2487 UV–VIS detector. All compounds were >95% pure. Spectroscopic data of compounds are listed below (^{1}H and ^{13}C NMR spectra are in the Supplementary material).

2.2. General procedure for Suzuki coupling reactions

2.2.1. 5-Phenylthiophene-2-carbaldehyde (3)[27]

To a dry glass flask purged with argon were added $Pd(OAc)_2$ (3.4 mg, 0.015 mmol), PPh_3 (16 mg, 0.06 mmol) and dry DME