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Flucytosine analogues obtained through Biginelli reaction as efficient combinative antifungal agents

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ABSTRACT

Invasive fungal infection is a problem that continues to challenge the healthcare sector. New antifungals and new therapeutic strategies are needed to address this challenge. We previously reported that the combination of a synthetic compound with a drug with known mechanism of action is a good strategy to treat aggressive and resistant fungi. Here we revisited our approach and synthesized structural analogues of flucytosine, which is a synthetic antifungal and is being studied for its use in combination therapy with other antifungal drugs. Pyrimidin-one and −thione (often known as DHPM's) as flucytosine analogues were obtained through a Biginelli reaction of corresponding aldehydes, ethylacetoacetate and urea/thiourea. Structure was confirmed by FTIR, ^{1}HNMR, ^{13}CNMR, COSY and MS (ESI^{+}) analysis. All the newly synthesized derivatives were evaluated for the antifungal activity alone and in combination of two most commonly used antifungal drugs, amphotericin B and fluconazole against different clinically isolated *Candida albicans* strains. Minimum inhibitory concentration results confirmed that BG4 possess high antifungal activity against all the tested strains (MIC = 1−32 μg/ml). For all the combinations with amphotericin B and fluconazole, 37% were synergistic followed by 30% additive and 24% indifferent interactions. Interestingly, 9% antagonistic interaction was observed when BG1 and BG3 were combined with fluconazole, however, no antagonistic interaction was observed with amphotericin B. In-depth studies of all the synergies were done by constructing isobolograms with nine different ratio combinations. These results warrant the use of DHPM derivatives as chemosensitising agents which could lower down the dosages of the antifungal drugs to treat invasive fungal diseases.

Opportunistic fungal infections are associated with increasing rates of mortality and morbidity, especially amongst immunocompromised patients [1]. Existing treatment options are limited and fungal infections are now recognised for killing as many people, worldwide, as tuberculosis and malaria [2]. Current antifungal agents show narrow spectrum of activity, poor bioavailability, toxicity, interactions with other drugs, or have fungistatic rather than fungicidal activity [3]. Emerging multiple drug resistance, particularly to azole class drugs, is now a serious issue in the treatment of fungal infections [4]. Thus, there is an urgent need to generate new, efficacious, non-toxic compounds with broad-spectrum antifungal activity. In this pursuit we previously reported on the synergetic interactions of some semi-synthetic analogues of eugenol with fluconazole and observed synergy (36%), additive (41%) or indifferent (23%) interactions; no antagonistic interactions were observed. Significant impairment of ergosterol biosynthesis coupled with down regulation of the important ergosterol biosynthesis pathway gene-*ERG11* was also followed in all *C. albicans* strains [5].

Despite the challenges in identifying new antifungal agents, considering our previous work in this field [5–7], it is our premise that revisiting and mimicking old antifungals could help in finding new antifungal leads. Advances in identifying new sources of antifungals and new strategies to treat fungal infections are providing chemical leads for new drugs [8,9]. As a new and effective strategy,

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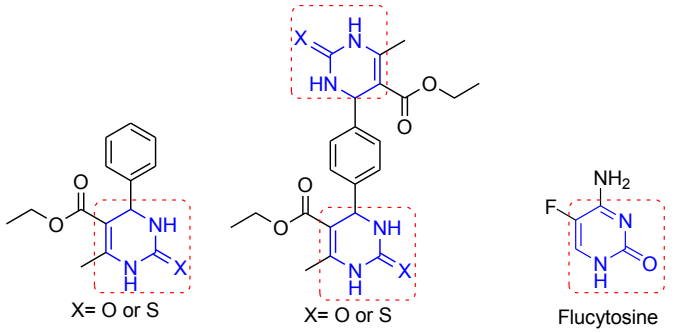


Fig. 1. DHPM analogues as structural mimics of Flucytosine.

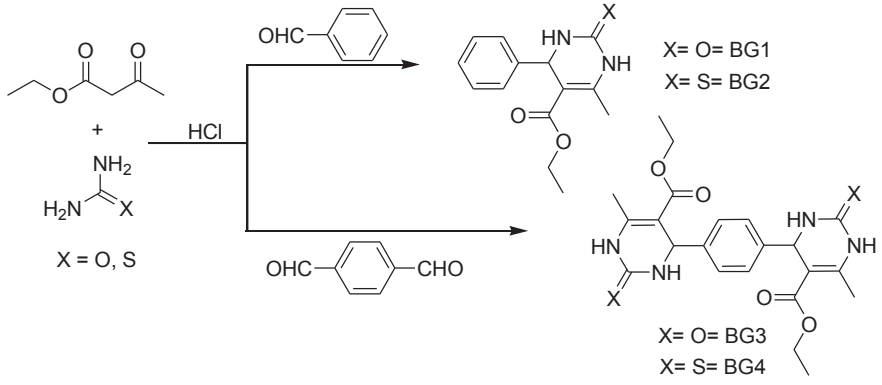
drug combinations have been used for the treatment of diseases like cancer, HIV or cardiovascular diseases and it is believed that drug combinations are better at controlling complex diseases with minimal resistance [10–12].

Flucytosine, a synthetic antimycotic compound has been receiving growing interest due to its use in combination with other antifungal agents, such as ketoconazole, fluconazole and itraconazole [13]. Combination of flucytosine with amphotericin B has been found to be a superior therapeutic option against opportunistic fungal pathogens such as *Candida albicans*, *Candida tropicalis* and *Cryptococcus neoformans* [14,15], but the incidence of side-effects of a combination therapy, particular with amphotericin B were found to be higher in certain cases. In this study we choose to mimic the structure of flucytosine and synthesize pyrimidinone/thione analogues, commonly known as DHPM's (in this manuscript we will refer to these compounds as DHPM derivatives) sharing a common basic ring skeleton with flucytosine through a Biginelli reaction (Fig. 1). Mono- and bis-DHPM derivatives (BG1-BG4) were synthesized to study the effect of two pyrimidin-one/thione rings in a single molecular scaffold (bia-analogues) on the antifungal efficacy, compared to the mono analogues as well as the synergetic interactions of these compounds with amphotericin B and fluconazole.

The synthetic potential of this heterocycle synthesis (Biginelli reaction) remained unexplored for quite some time. In the 1970's and 1980's interest slowly increased, and the scope of the original cyclocondensation reaction shown in Scheme 1 was gradually extended by variation of all three building blocks, allowing access to a large number of multifunctionalized dihydropyrimidines [16,17]. Especially in the last two decades, the chemistry and biology of 3,4-dihydropyrimidin-2(1H)-ones (or -thiones), also referred to as DHPMs, have experienced a return to prominence. Because of their already known biological activities as calcium channel modulators, mitotic kinesin inhibitors, adrenergic receptor antagonists, antibacterials, antifungals, antivirals, and others [16–22]. Some DHPM derivatives (Fig. 2) have attracted much attention and interest of many research groups, mainly considering the possibility of diversity generation and direct access to new libraries of bioactive compounds.

DHPM derivatives (BG1-BG4) were obtained through a one pot Biginelli reaction of corresponding aldehydes, ethyl acetoacetate and urea or thiourea in refluxing ethanol with a catalytic amount of HCl (Scheme 1). All the compounds were obtained in 70–80% yield and recrystallized from appropriate solvents, and were found to be soluble in methanol, ethanol, DMSO, DMF, acetonitrile, dichloromethane, and chloroform. Structure of all the synthesized compounds was established by FTIR, ^{1}H NMR, ^{13}C NMR, COSY and ESI-MS spectral analysis. IR spectra of the compounds show characteristic absorption band at 3229-3325 cm^{-1} for NH stretch, 1721–1661 for C=O and 1694–1568 for C=O (amide), and C=S (thioamide) groups. The ^{1}HNMR data of all compounds exhibit characteristic peaks at 7.71–10.31 ppm for NH protons and a multiplet in the region of 7.21–7.37 ppm due to the presence of aromatic protons. Sharp singlet at 2.09 ppm is a characteristic of the methyl protons of all the pyrimidin-one/thione derivatives BG1-BG4. The benzylic proton CH (H-4) appeared as a doublet around 5.15–5.17 ppm due to its coupling with the adjacent NH (H-3) proton. However, in all cases, the NH (H-3) proton appeared as a broad singlet around 7.71–9.63 ppm due to poor resolution (See Fig. S1 for structure). Another NH (H-1) also appeared as a singlet in all the spectra. The other signals and peaks of the IR and ^{1}H NMR are in complete agreement with the assigned structures. ^{13}C NMR exhibited peaks characteristic for the compounds. The mass spectra of the compounds displayed a molecular ion peak at appropriate m/z values, which corresponded well with the respective molecular formula. The COSY spectrum of BG1 and BG3 show similar coupling interactions for all the derivatives between NH and CH protons of the pyrimidinone ring besides coupling of CH_3 and CH_2 protons of the ester linkage (Fig. S1 in supporting information). The detailed spectra are given in supporting information.

All the four newly synthesized compounds have been evaluated for the antifungal activity against eight different strains of *C. albicans*, following the standard protocol of CLSI [23]. Clinical strains were isolated from HIV positive patients from the Charlotte Maxeke Johannesburg Academic Hospital, Johannesburg, South Africa and where identified using CHROMagar media (CHROMagar Paris, France). Of the four compounds (BG1-BG4) tested, BG4 was the most active compound against all the tested strains including the clinical isolates with the MIC values ranging between 1 and



Scheme 1. Synthesis of DHPM derivatives BG1-BG4 using Biginelli reaction conditions.