Microbial Pathogenesis 105 (2017) 57–62

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Flucytosine analogues obtained through Biginelli reaction as efficient combinative antifungal agents

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ARTICLE INFO

Article history:
Received 25 November 2016
Received in revised form
3 February 2017
Accepted 4 February 2017
Available online 9 February 2017

Keywords:
Pyrimidinone
Flucytosine analogues
Biginelli reaction
Antifungal
Synergy

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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http://dx.doi.org/10.1016/j.micpath.2017.02.006
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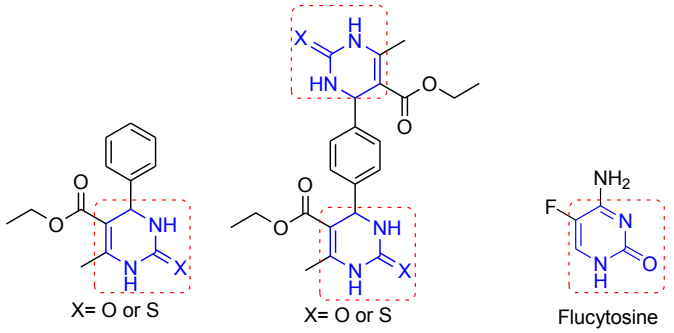


Fig. 1. DHPM analogues as structural mimics of Flucytosine.

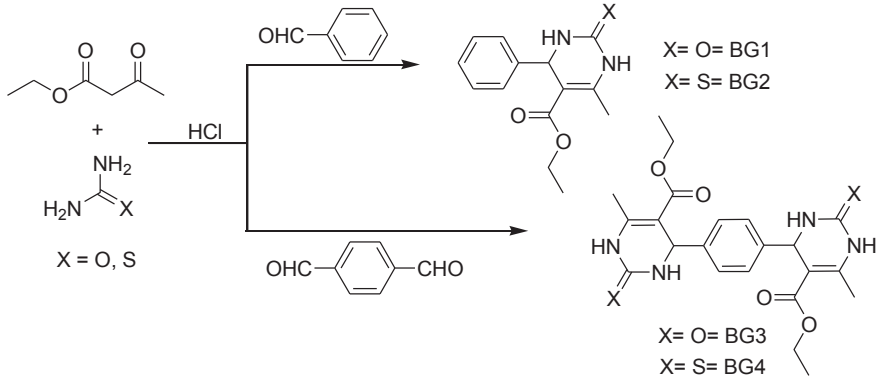
drug combinations have been used for the treatment of diseases like cancer, HIV or cardiovascular diseases and it is believed that drug combinations are better at controlling complex diseases with minimal resistance [10–12].

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.

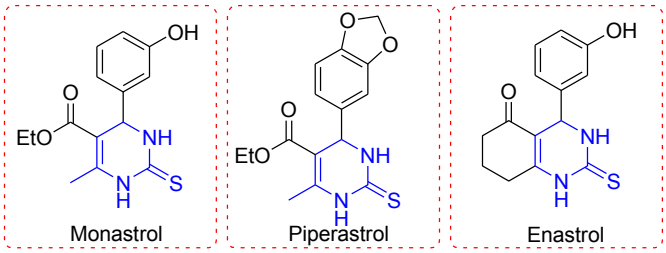


Fig. 2. Some examples of known biologically active dihydropyrimidinones (DHPMs).

32 μg/ml (Table 1). BG4 was closely followed by BG3 with the MIC values ranging between 4 and 125 μg/ml against all the tested strains. Although, BG1 and BG2 also showed prominent antifungal activity against few strains but have moderate antifungal activity against most of the tested strains with MIC values ranging between 32 and 500 μg/ml and 16–500 μg/ml, respectively (Table 1). As expected, all the strains showed high susceptibility to both the antifungal drugs with MIC values ranging from 1–2 and 4–32 μg/ml for amphotericin B and fluconazole, respectively. The difference in activity could be attributed to the structure of the compounds; BG3 and BG4 are bis-derivatives, where as BG1 and BG2 contain a single pyrimidinone (BG1) and pyrimidithione (BG2) ring in their skeleton respectively. The activity of the bis-analogues is further governed by the presence of sulphur as thioamide group in BG4 in place of oxygen in BG3, where BG4 showed enhanced activity compared to BG3. However, in any case the bis-analogues were more active than their corresponding mono-analogues, which lead to say that the presence of similar bioactive rings in a single molecular scaffold enhances its biological profile. This enhancement could be due to strong interactions (hydrogen bonding, Van der Waals or hydrophobic) of the bis-analogue with the target, which results in the enhancement of the activity. Presence of thione sulphur (C=S) in BG4 in place of keto oxygen (C=O) in BG3 may also be a reason of higher activity possibly due to the higher nucleophilic character of sulphur, due to its large size, which makes it readily polarizable, and its lone pairs of electrons readily accessible. The possible mechanism of action of these flucytosine mimics could be similar to one of the proposed mechanism of action of flucytosine, where it acts as a potent inhibitor of thymidylate synthetase, which is a key enzyme in the biosynthesis of DNA. Since thymidylate synthetase is a crucial source of thymidine, its deficiency consequently leads to fungal DNA synthesis inhibition [13]. These propositions however demand a detailed study into the mechanism of action of these derivatives to establish the point.

The limited use of flucytosine monotherapy, because of the acquired resistance, impetus the combinational therapies. With the consequence, we perpetuate the combinational interaction of these flucytosine mimics with the two commonly used antifungal drugs, amphotericin B and fluconazole. Combinational therapies are the first-line treatment for disseminated fungal infections, especially with the immunocompromised and immunosuppressed patients [24]. To determine the combinational interaction of the compounds BG1-BG4 with amphotericin B and fluconazole in 1:1 ratio, fractional inhibitory concentration index (FICI) was determined for all the combinations. Out of all the combinations (n = 64) between the test compounds with amphotericin B and fluconazole, 37% (24) were synergistic interactions, 30% (19) were additive interactions, 24% (15) were indifferent interactions and 9% (6) were antagonistic interactions (Table 2). Most of the synergistic interactions (12 out of 16) were observed for BG4 with amphotericin B and fluconazole, while no synergistic combination was observed for BG1, BG2 and BG3 showed 56% and 19% synergistic interactions between all the combinations. Both BG1 and BG4 showed 25% additive interaction while BG2 and BG3 showed 19% and 50% additive interactions respectively. BG1 showed most of the indifferent combinations (9 out of 16) while no indifferent combination was observed for BG4 against any of the tested *C. albicans* strains. Both BG1 and BG3 showed 19% of antagonistic interactions when combined with fluconazole. However no antagonistic interaction was observed with BG2 and BG4. Of all the combinations, the most significant enhancement of antifungal activity was observed between BG4 and amphotericin B (FICI = 0.124) against *C. albicans* 002B1. Amphotericin B showed predominantly favourable interactions, synergistic in 15 out of 32 combinations. Fluconazole contrastingly showed synergy with only 9 out of 32 combinations. Interestingly, all the 6 antagonistic interactions were observed with fluconazole.

For combinational therapies, one big drawback is to formulate the appropriate concentration of the individual components. It is very rare that 1:1 combination will generate the best of the formulations for chemosensitising potential of the antifungal drugs. Also, it has been previously reported that the result interpretation based on the FICI values has some important disadvantages [25]. Based on these facts; we studied the individual components, showing synergy in 1:1 ratios, in a range of different ratios and plotted the isobolograms from the results.

FIC_A represents MIC of DHPM derivative when combined with the drug while as FIC_B is MIC of the drug when combined with DHPM derivative. INT: Interpretation, SYN: synergy, ADD: additive, IND: indifferent, ANT: antagonism.

For in-depth studies of the synergistic interactions observed in 1:1 ration all the DMAP derivatives BG1-BG4 were combined with the antifungal drugs in nine different ratios and isobolograms were constructed. Representative isobolograms for compounds BG2, BG3 and BG4 when combined with amphotericin B and fluconazole are shown in Fig. 3. From these isobolograms it is evident that most of the combinations, irrespective of the combined ratios, are within the 0.5 values and therefore represent synergistic interaction while only few of these ratios belong to 0.5–1.0 values depicting additive interactions. No indifferent or antagonistic interaction was observed with any of the combined ratio. From the isobolograms, it is evident that BG4 had a broadest synergism when combined with amphotericin B against *C. albicans* ATCC90028, where all the nine combined ratios showed synergy. This has been followed by BG2 when combined with fluconazole against *C. albicans* 003gr where eight of the combined ratios showed synergy and one ratio showed additive effect.

There is large body of clinical experience with the combination of amphotericin B and flucytosine to treat invasive fungal infections [25,26]. Also some studies reported the antagonistic interaction of fluconazole when combined with flucytosin [25–27]. However, with these flucytosin mimics (DHPM derivatives BG1-BG4), only 9% of the total combinations showed antagonisim when combined

Table 1
Minimum inhibitory concentrations (μg/ml) of four newly synthesized Biginelli series compounds along with amphotericin B (AmB) and fluconazole (FLC), against various *C. albicans* strains.

Strains (*C. albicans*)	MIC (μg/ml)					
	BG1	BG2	BG3	BG4	AMB	FLC
ATCC 90028	64	32	16	2	2	4
074	125	125	32	2	2	8
072gr	250	125	125	32	2	16
002B1	32	16	4	1	1	4
003gr	250	64	32	8	2	4
004gr	500	500	125	32	2	32
004B1	125	32	8	1	1	8
0079gr	125	64	32	4	2	16

Table 2
The fractional inhibitory concentration index of DHPM derivatives (BG1-BG4) tested in 1:1 combinations with fluconazole and amphotericin B.

Strains		BG1 + FLC	INT	BG1 + AmB	INT	BG2 + FLC	INT	BG2 + AmB	INT	BG3 + FLC	INT	BG3 + AmB	INT	BG4 + FLC	INT	BG4 + AmB	INT
90028	FIC_A	0.250	ANT	0.032	IND	0.062	ADD	0.015	SYN	0.125	ADD	0.062	ADD	0.250	SYN	0.125	SYN
	FIC_B	4.000		1.000		0.500		0.250		0.500		0.500		0.125		0.125	
	∑FIC	4.250		1.032		0.562		0.265		0.625		0.562		0.375		0.250	
074	FIC_A	0.256	ANT	0.008	ADD	0.032	ADD	0.004	SYN	1.000	ANT	0.032	ADD	0.250	SYN	0.250	SYN
	FIC_B	4.000		0.500		0.500		0.250		4.000		0.500		0.062		0.250	
	∑FIC	4.256		0.508		0.532		0.254		5.000		0.532		0.312		0.500	
072 gr	FIC_A	0.125	IND	0.016	IND	0.256	IND	0.032	IND	0.125	IND	0.008	ADD	0.250	ADD	0.015	SYN
	FIC_B	2.000		2.00		2.000		2.000		1.000		0.500		0.500		0.250	
	∑FIC	2.125		2.016		2.256		2.032		1.125		0.508		0.750		0.265	
002 B1	FIC_A	0.062	ADD	0.015	ADD	0.062	SYN	0.015	SYN	4.000	ANT	0.062	SYN	0.250	SYN	0.062	SYN
	FIC_B	0.500		0.500		0.250		0.250		4.000		0.250		0.062		0.062	
	∑FIC	0.562		0.515		0.312		0.265		8.000		0.315		0.312		0.124	
003 gr	FIC_A	0.032	IND	0.016	IND	0.015	SYN	0.008	SYN	0.062	ADD	0.031	ADD	0.250	ADD	0.062	SYN
	FIC_B	2.000		2.000		0.250		0.250		0.500		0.500		0.500		0.250	
	∑FIC	2.032		2.016		0.265		0.258		0.562		0.531		0.750		0.312	
004 gr	FIC_A	0.032	ADD	0.004	IND	0.021	SYN	0.001	SYN	0.064	SYN	0.004	SYN	0.062	SYN	0.015	SYN
	FIC_B	0.500		1.000		0.250		0.250		0.250		0.250		0.062		0.250	
	∑FIC	0.532		1.004		0.266		0.251		0.314		0.254		0.124		0.265	
004 B1	FIC_A	0.128	IND	0.008	IND	0.250	IND	0.015	ADD	0.500	ADD	0.062	ADD	0.500	ADD	0.500	ADD
	FIC_B	2.000		1.000		1.000		0.500		0.500		0.500		0.062		0.500	
	∑FIC	2.128		1.008		1.250		0.515		1.000		0.562		0.562		1.000	
0079 gr	FIC_A	0.500	ANT	0.016	IND	0.250	IND	0.008	SYN	1.935	ANT	0.062	IND	0.252	SYN	0.125	SYN
	FIC_B	3.906		1.000		1.000		0.250		3.906		1.000		0.060		0.250	
	∑FIC	4.406		1.016		1.250		0.258		5.859		1.062		0.312		0.375	

with fluconazole and no antagonistic interaction was observed with amphotericin B. Despite the positive synergistic interactions of these DHPM derivatives with the known antifungal drugs, monotherapy of these compounds will not be recommended, with the fact that use of the parent drug, flucytosine, is very prone to the acquired drug resistance when used alone [28]. Also to avoid the risk of influencing significance, we advocate the use of DHMP derivatives with amphotericin B. However, further studies using drug resistant strains and *in vivo* studies will warrant the use of these flucytosine mimics as antifungal drugs to treat invasive fungal infections.

In conclusion mono and bis-DHPM derivatives as flucytosine mimics were synthesized under Biginelli reaction conditions with an aim to check the synergetic interactions of these derivatives

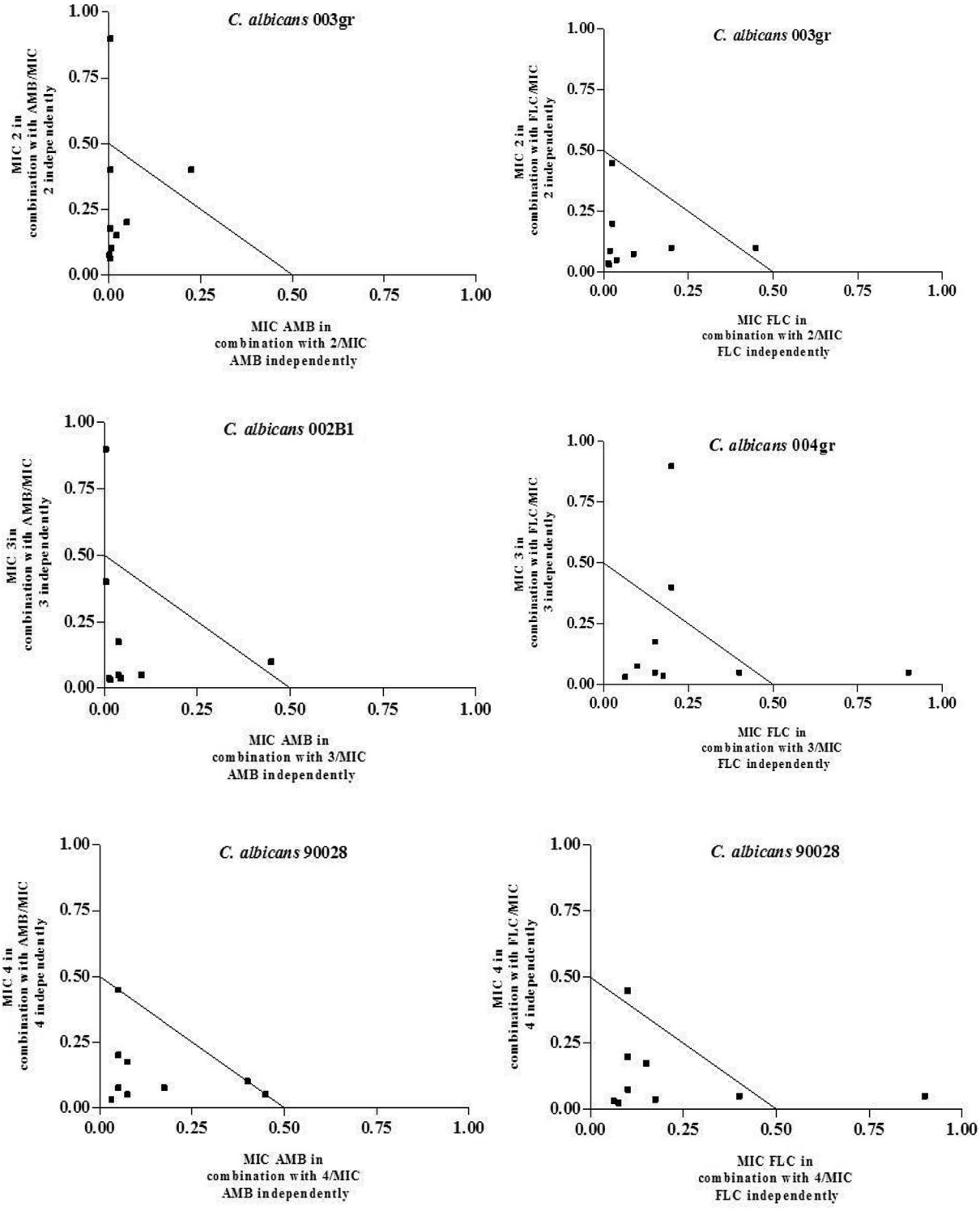


Fig. 3. Representative isobolograms for the synergistic interactions of BG2 (2), BG3 (3) and BG4 (4) with amphotericin B (AMB) and fluconazole (FLC) in nine different ratios against different *Candida albicans* isolates.

with the known antifungal drugs amphotericin B and fluconazole. The structure of these DHPM analogues had a marked effect on the antifungal efficacy and synergy, where bis-derivatives were found to be more efficacious than their corresponding mono analogues. BG4 derivative with two pyrimidithione rings in a single molecular scaffold showed high synergy with amphotericin-B and fluconazole both followed by BG3, BG2 and BG1. These studies guide us further to revisit the structures of old antifungal drugs to synthesize structure mimics, which could probably show better antifungal efficacy or synergy with the known antifungal drugs in treating more invasive fungal infections with less resistance and toxicity. The structure of the DHPM derivatives used in this study is being

further optimized to obtain lead molecules, which can be used in combination therapy for the treatment of fungal infections.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by Fundo Europeu de Desenvolvimento Regional-QREN- COMPETE through project PTDC/AAC-CLI/118092/2010 of Fundação para a Ciência e a Tecnologia (FCT) and FCT Postdoctoral grants SFRH/BPD/86581/2012 (M.Y. Wani) and SFRH/BPD/86507/2012 (S. Kumar). We also thank Centro de Quimica de Coimbra (CQC), FCTUC for their support. CQC is funded through Project PEst-OE/QUI/UI0313/2014. Authors thank Department of Oral Biological Sciences, University of the Witwatersrand, for the clinical strains.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2017.02.006.

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