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Repurposing antipsychotic drugs into antifungal agents: synergistic combinations of azoles and bromperidol derivatives in the treatment of various fungal infections

Selina Y. L. Holbrook,[a] Atefeh Garzan,[a] Emily K. Dennis,[a] Sanjib K. Shrestha,[a] and Sylvie Garneau-Tsodikova[a,*]

[a]*Department of Pharmaceutical Sciences, University of Kentucky, 789 South Limestone Street, Lexington, KY, 40536-0596, USA*

(*Corresponding author's email: sylviegtsodikova@uky.edu; Phone: 859-218-1686; Fax: 859-257-7585)

Running title: Antifungal drug combinations

ABSTRACT

As the number of hospitalized and immunocompromised patients continues to rise, invasive fungal infections, such as invasive candidiasis and aspergillosis, threaten the life of millions of patients every year. The azole antifungals are currently the most prescribed drugs clinically that display broad-spectrum antifungal activity and excellent oral bioavailability. Yet, the azole antifungals have their own limitations and are unable to meet the challenges associated with increasing fungal infections and the accompanied development of resistance against azoles. Exploring combination therapy that involves the current azoles and another drug has been shown to be a promising strategy. Haloperidol and its derivative, bromperidol, were originally discovered as antipsychotics. Herein, we synthesize and report a series of bromperidol derivatives and their synergistic antifungal interactions in combination with a variety of current azole antifungals against a wide panel of fungal pathogens. We further select two representative combinations and confirm the antifungal synergy by performing time-kill assays. Furthermore, we evaluate the ability of selected combinations to destroy fungal biofilm. Finally, we perform mammalian cytotoxicity assays with the representative combinations against three mammalian cell lines.

Keywords: Biofilm, *Candida albicans*, Cytotoxicity, Drug synergy, Filamentous fungus, Time-kill

INTRODUCTION

Fungal infections are an ever-growing global health problem. Even though fungal infections can affect both the healthy and the sick, they pose a much greater threat to chronically ill and immunocompromised patients [1]. As the number of hospitalized and immunocompromised patients increases, the rise of fungal infections and the associated resistance problems are raising alarm. *Candida albicans* is a leading cause of fungal infections and accounts for up to 70% of total incidents worldwide [2]. Invasive candidiasis, a bloodstream infection of *Candida* species, is one of the most common nosocomial fungal diseases with an estimate of 350,000 incidents worldwide every year and a 30-55% mortality rate [3, 4]. From 2000 to 2005, the number of invasive candidiasis in the USA rose by 52% and inevitably much more in less developed countries, such as Brazil and India. In addition to *Candida* species, some filamentous fungi, such as *Aspergillus*, also cause life-threatening infections. If left untreated, invasive aspergillosis can result in a 99% mortality rate, and therefore, is another fungal infection that is calling for immediate attention worldwide. For instance, the increasing use of corticosteroid in 4.8 million asthma patients is linked to over 400,000 patients developing chronic pulmonary aspergillosis [5].

Fungi infect not only humans but also various food sources. From the Irish potato famine in the 19th century [6, 7] to today's spread of *Puccina graminis tritici* Ug99, which is responsible for stem or black rust diseases on wheat [8], fungal infections have never been solely a burden to clinical healthcare providers, but also to food production and quality control professionals. By infecting crops and livestock, fungi not only result in severe damage in the food production industry, but can be spread to humans through food and cause diseases.

The challenges we face with fungal infections are evident and urgent, yet, our repertoire of antifungals is limited [3]. Currently, there are only few antifungal drugs for the treatment of systemic infections, including polyenes (*e.g.*, amphotericin B (AmB)), azoles (*e.g.*, fluconazole (FLC), itraconazole (ITC), ketoconazole (KTC), posaconazole (POS), and voriconazole (VOR); Fig. 1A), and echinocandins (*e.g.*, caspofungin (CAS)), each of which has severe limitations. The intravenous drug AmB was the first antifungal agent approved for systemic use for invasive fungal infections over 50 years ago [9]. However, its dose-limiting toxicity and other adverse

effects often result in interruption of treatment courses [10]. Another intravenous antifungal drug family, the echinocandins, was shown to possess lower toxicity and to be better tolerated than AmB in various formulations [3]. The broad-spectrum nature of the echinocandins has made them a better alternative to AmB for treating invasive fungal infections.

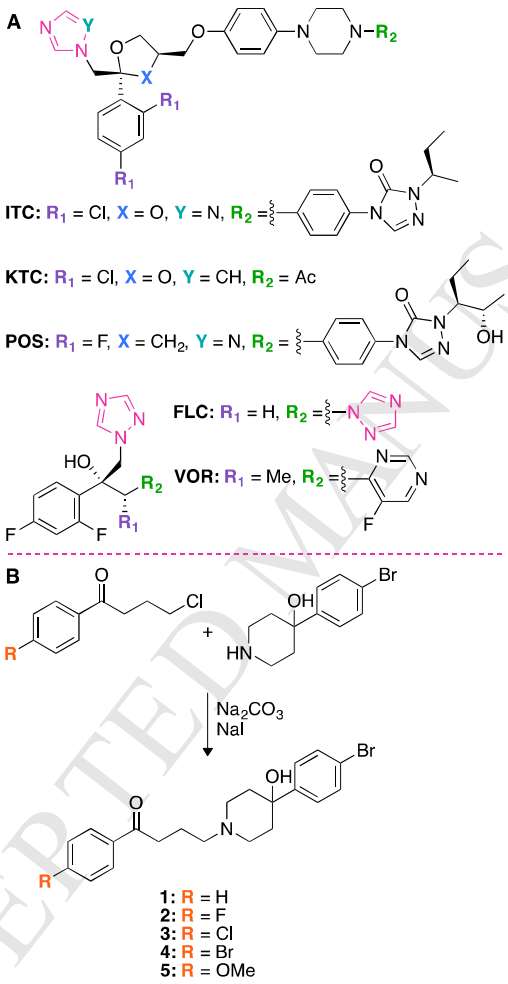

Fig. 1. Structures of **A.** the common azole antifungals with the triazole pharmacophore highlighted in pink as well as **B.** the chemical synthesis of bromperidol series compounds (**1-5**) involved in this study.

The azoles are the only class of oral antifungals identified due to their excellent oral bioavailability [11, 12]. By targeting the sterol 14α-demethylase, azoles inhibit the biosynthesis of ergosterol, which is a vital component of the fungal cell membrane [13, 14]. Inhibition of this enzyme causes the methylated sterol side products to accumulate inside the fungal cells, which is toxic to fungi and results in cell death. In addition to excellent bioavailability, the azole

antifungals also exhibit fewer adverse effects than AmB. Consequently, the azole antifungals quickly became the most clinically prescribed antifungal class worldwide since their introduction to the market. However, azoles have their own limitations. Azoles inhibit cytochrome P450 enzymes, which results in undesired interference with the metabolism of numerous other drugs, making it difficult for patients being treated with multiple concurrent medications [15]. Moreover, azoles are often found to be fungistatic towards many fungi, resulting in only temporary inhibition of fungal growth [16]. The lack of fungicidal activity has made it challenging to prevent fungal regrowth and the accompanied development of antimicrobial resistance to azoles.

Due to the clinical importance of azole antifungals, many research groups have tried to develop new azole antifungal therapies with broader antifungal spectra, reduced undesired drug-drug interactions, and diminished other adverse effects. Besides developing new azole derivatives [17-19], exploring various combination therapies that work synergistically with the current azole antifungals has proved fruitful [20-22]. For instance, various azoles were shown to display synergistic antifungal activity with a series of amphiphilic tobramycin derivatives with azoles inhibiting ergosterol biosynthesis and the tobramycin derivatives proposed to act on the fungal membrane integrity [23, 24]. Sertraline, an antidepressant, was also found to work synergistically with FLC against cryptococcal infections [25].

The benefits of repurposing existing drugs that have already been approved by the US Food and Drug Administration (FDA) for a new application against fungal infections extend beyond yielding potentially new antifungal therapies. The previous evaluations of the approved drugs from years of clinical studies can also provide valuable information about these drugs, such as their pharmacokinetics, pharmacodynamics, metabolism, and toxicity profiles. In the effort of repurposing existing drugs, several non-antifungal drugs were identified with antifungal activity and were summarized in a previous review article [26]. Haloperidol (trade name Haldol) is an FDA-approved oral antipsychotic that was recently discovered to possess antifungal activity towards a drug-sensitive *C. albicans* strain (*C. albicans* SC5314) [27]. Bromperidol, a haloperidol derivative also with antipsychotic properties, was reported to kill mycobacteria in a synergistic manner with spectinomycin [28], further suggesting that this antipsychotic drug may

possess antimicrobial properties. Although the cellular antifungal target of haloperidol/bromperidol is still in debate, this antipsychotic drug can potentially be developed into a combination therapy with azoles as a new antifungal strategy.

Herein, we synthesized a series of bromperidol (**2**) and a series of its derivatives (**1** and **3**-**5**) (Fig. 1B) and evaluated the combinational antifungal properties of these compounds with a variety of clinical azole antifungal drugs. We tested the combinations of five azoles (FLC, ITC, KTC, POS, and VOR) and compounds **1-5** against a wide selection of fungal strains, including seven *C. albicans*, one non-*albicans Candida* (*C. glabrata*), and one filamentous fungus (*A. terreus*). After assessing the synergistic effect between the azole antifungals and compounds **1-5**, we further performed time-kill assays to evaluate this antifungal strategy in a time- and dose-dependent manner. Moreover, we evaluated the combination of bromperidol (**2**) and selected azole antifungals for its ability to disrupt yeast biofilm in a representative *C. albicans* strain. Finally, we performed mammalian cytotoxicity assay with three selected mammalian cell lines in order to estimate the mammalian cytotoxicity exerted by the combinations of compounds **1-5** and azole antifungals.

RESULTS AND DISCUSSIONS

Chemical synthesis of bromperidol and its derivatives

In this study, we aim to evaluate the antifungal activity of the combination of common azole antifungals (FLC, ITC, KTC, POS, and VOR, Fig. 1A) and bromperidol (**2**) as well as its derivatives (**1** and **3**-**5**) (Fig. 1B) against nine fungal pathogens. Each of the nine fungal strains tested in this study also presents distinct biology and a complex resistance profile. The bromperidol series compounds (**1**-**5**) were synthesized by a reaction of 4-(4-bromophenyl)-4-hydroxypiperidine with different 4-chlorobutyrophenone derivatives in the presence of sodium iodide and sodium carbonate (Fig. 1B). The substituents at the R position of the five compounds varied in size and included a hydrogen atom, halogens (*e.g.*, fluorine, chlorine, and bromine), and a methoxy group. Detailed synthetic procedures and characterization for each compound are provided in the Supporting Information.

Antifungal synergy of the combinations of azole antifungals and bromperidol series

compounds 1-5 by checkerboard assays

Prior to evaluating the combinational antifungal effect of azoles and compounds **1-5**, we first determined the minimum inhibitory concentration (MIC) values of each drug against a variety of fungal pathogens to examine their innate antifungal effect and better gauge for an appropriate concentration range to use for the following checkerboard assay (Tables 1 and 3, displayed as MIC_{alone}). We first selected two representative *C. albicans* strains (strains *B* and *F*) (Table 1). *C. albicans* ATCC 10231 (strain *B*) is sensitive to most azoles whereas *C. albicans* ATCC 64124 (strain *F*) displays resistance to most azoles tested. The MIC values of the azole antifungals against some fungal strains determined in this study were in agreement with previously reported values [17]. Furthermore, we observed no antifungal effect for compounds **1-5** when tested alone.

We then tested the synergistic antifungal effects of the five azoles and compound **1-5** in combination by checkerboard assays and calculated the fractional inhibitory concentration index (FICI) against strains *B* and *F* (Table 1). The FICI cutoff values for determining synergy are: synergistic (SYN) if FICI ≤0.5, additive (ADD) if 0.5 < FICI ≤ 4, antagonistic (ANT) if FICI >4 [29]. In some cases where the FICI was >0.5, however, we observed a significant decrease in the MIC values of at least one of the drugs in the combinations. In such cases, we further defined partial synergy (pSYN) as 0.5 < FICI ≤ 0.75 (indicating that both drugs showed reduction in MIC values and one drug showed ≥two-fold reduction in MIC value), and strong additive effect (ADD*) where one drug showed ≥two-fold reduction in MIC value. We deemed defining these two categories necessary, as with these combinations, we would still be able to use low amount of azoles in combination to achieve a similar antifungal effect as using high concentrations of azoles alone, which would alleviate azole-induced toxicity and side effects. One thing to note is that due to the resistant nature of some fungal strains and the insufficient antifungal activity of the tested drugs, we were unable to achieve full inhibition of fungal growth with some drugs, therefore, resulting in unbound MIC values such as >32 μg/mL for most azoles and >128, >64, and >32 μg/mL for most bromperidol derivatives. In these cases, we considered the MIC values to be 32 μg/mL for the azoles and 128, 64, and 32 μg/mL for compounds **1-5**, respectively, in order to calculate a bound FICI value. However, this approximation would produce overestimated FICI values that are higher than the true FICI values if the real MIC values could

be determined. Hence, the amount of synergy observed in this study (both in terms of the FICI value for each combination and in terms of the percentage of combinations with synergy) would be an underestimation of the real potential synergy.

Table 1. The combinational effect of various azoles and compounds **1-5** against two representative *C. albicans* strains.

Cpd	Azole	Strain	MIC alone (μg/mL)		MIC in combo (μg/mL)		FICI	Interp.	Cpd	Azole	Strain	MIC alone (μg/mL)		MIC in combo (μg/mL)		FICI	Interp.
			Azole	Cpd	Azole	Cpd						Azole	Cpd	Azole	Cpd		
1	FLC	*B*	>32	>64	32	64	2.00	ADD	**4**	FLC	*B*	>32	>64	>32	>64	2.00	ADD
		F	>32	>64	>32	>64	2.00	ADD			*F*	>32	>64	>32	>64	2.00	ADD
	ITC	*B*	1	>64	0.5	64	1.50	ADD		ITC	*B*	1	>64	0.5	64	1.50	ADD
		F	>32	>64	>32	>64	2.00	ADD			*F*	>32	>64	>32	>64	2.00	ADD
	KTC	*B*	1	>64	1	>64	2.00	ADD		KTC	*B*	2	>64	1	1	0.52	pSYN
		F	8	>64	4	32	1.00	ADD			*F*	8	>64	4	1	0.52	pSYN
	POS	*B*	1	>64	0.5	16	0.75	pSYN		POS	*B*	1	>64	0.5	32	1.00	ADD
		F	>32	>64	2	8	0.19	SYN			*F*	>32	>64	4	16	0.38	SYN
	VOR	*B*	1	>64	0.5	8	0.63	pSYN		VOR	*B*	1	>64	0.5	32	1.00	ADD
		F	>32	>64	8	32	0.75	pSYN			*F*	>32	>64	4	32	0.63	pSYN
2	FLC	*B*	>32	128	32	64	1.50	ADD	**5**	FLC	*B*	>32	>128	>32	>128	2.00	ADD
		F	>32	128	32	64	1.50	ADD			*F*	>32	>128	>32	>128	2.00	ADD
	ITC	*B*	1	128	0.25	64	0.75	pSYN		ITC	*B*	1	>128	1	>128	2.00	ADD
		F	>32	128	2	64	0.56	pSYN			*F*	>32	>128	>32	>128	2.00	ADD
	KTC	*B*	1	>128	0.5	32	0.75	pSYN		KTC	*B*	1	>128	1	>128	2.00	ADD
		F	16	>128	4	64	0.75	pSYN			*F*	8	>128	4	64	1.00	ADD
	POS	*B*	0.5	128	0.125	64	0.75	pSYN		POS	*B*	1	>128	1	>128	2.00	ADD
		F	>32	128	4	4	0.16	SYN			*F*	>32	>128	2	8	0.13	SYN
	VOR	*B*	0.5	128	0.25	32	0.75	pSYN		VOR	*B*	1	>128	0.5	32	0.75	pSYN
		F	>32	128	4	8	0.19	SYN			*F*	>32	>128	8	128	1.25	ADD*
3	FLC	*B*	>32	>32	32	16	1.50	ADD										
		F	>32	>32	>32	>32	2.00	ADD										
	ITC	*B*	1	>32	1	>32	2.00	ADD										
		F	>32	>32	>32	>32	2.00	ADD										
	KTC	*B*	2	>32	0.5	16	0.75	pSYN										
		F	8	>32	4	0.5	0.52	pSYN										
	POS	*B*	1	>32	0.5	4	0.63	pSYN										
		F	>32	>32	2	4	0.19	SYN										
	VOR	*B*	1	>32	0.25	16	0.75	pSYN										
		F	>32	>32	4	16	0.63	pSYN										

Strains: *B* = *C. albicans* ATCC 10231, *F* = *C. albicans* ATCC 64124.
The FICI cutoff values for determining synergy are: synergistic (SYN) if FICI ≤ 0.5, additive (ADD) if 0.5 < FICI ≤ 4, antagonistic (ANT) if FICI > 4.
Note: Where the highest concentration of a compound or azole drug alone did not achieve optical growth inhibition, the MIC_{alone} value used in the FICI calculation is the highest concentration tested of that compound or azole drug.
Indicates synergy (SYN, both drugs showed ≥2-fold reduction in MIC value).
Indicates partial synergy (pSYN, both drugs showed decrease in MIC value and one drug showed ≥2-fold reduction in MIC value).
Indicates strong additive effect (ADD*, only one drug showed ≥2-fold reduction in MIC value).
Indicates weak additive effect (ADD, neither of the drugs showed ≥2-fold reduction in MIC value).

Of the 25 combinations tested against strains *B* and *F* in the first round, we found six combinations (24% of all combinations) to be synergistic with FICI values ranging from 0.13 to 0.5 against the azole-resistant strain *F*. The best combination with the lowest FICI value of 0.13 observed was compound **5** and POS, which showed decrease of MIC_{alone} of azole and compound **5** from >32 and >128 μg/mL to 2 and 8 μg/mL (16-fold reduction in MIC values for both drugs), respectively. The second best combination discovered with an FICI value of 0.16 was with

compound **2** and POS, which showed decrease of MIC_{alone} of azole and compound **2** from >32 and 128 μg/mL to 4 (8-fold MIC reduction) and 4 μg/mL (32-fold MIC reduction), respectively. In these cases, we demonstrated that the combinations of azoles and bromperidol derivatives could synergistically inhibit the growth of an azole-resistant *C. albicans* that otherwise would not have responded to high concentrations of either drugs given in the assay. Amongst the six combinations that display synergy, four of them (compound **1** with POS, compound **2** with POS or VOR, and compound **3** with POS) were also found to display partial synergy ($0.5 < FICI \leq 0.75$) against strain *B*. Moreover, partial synergy was also observed against both strains *B* and *F* for the following combinations: compound **1** with VOR, compound **2** with ITC or KTC, compound **3** with KTC or VOR, and compound **4** with KTC. For example, with the combination of compound **2** and ITC against strain *F* (FICI = 0.56), inhibition of fungal growth could be achieved with 2 μg/mL of ITC (16-fold MIC reduction) and 64 μg/mL of compound **2** (1-fold MIC reduction). Partial synergy was also detected for compound **4** with VOR against strain *F* as well as for compound **5** with VOR against strain *B*. With the FICI values calculated in this study likely to be overestimated as explained above, these combinations with partial synergy also have great potentials for being developed into synergistic antifungal therapies.

Besides the combinations found to exert synergistic or partial synergistic effect between the azole antifungals and our bromperidol derivatives, we also found one combination to display strong additive effect, which is the combination of VOR and compound **5** against strain *F*. An FICI value of 1.25 indicated that this combination displayed additive effect. However, looking at the MIC values of each drug alone and in combination, we found a significant decrease (4-fold reduction) in the MIC of VOR in combination compared to that of VOR alone even though the MIC value of compound **5** showed no further decrease from 128 μg/mL. This suggested that the addition of compound **5** significantly reduced the amount of VOR required to inhibit fungal growth, alleviating the toxicity and other undesired side effects of azole antifungals. The rest of the combinations (11 out of 25 combinations) displayed weak additive effect against both strains *B* and *F* with FICI values ranging from 1.00 to 2.00. No antagonism was found in any combinations tested in this study.

When we collected all FICI values for these 25 combinations in Table 2 and analyzed them in a heat map style table, it became clear that all synergistic combinations discovered so far involve either POS or VOR. Of the five combinations involving POS, 100% displayed synergy against the azole-resistant *C. albicans* strain *F*, and 60% displayed partial synergy against strain *B*. Of all the combinations involving VOR, only one combination (that with compound **2**) was found to be synergistic against strain *F*. Meanwhile, four combinations with VOR showed partial synergy against strains *B* (compounds **1-3** and **5**) and *F* (**1**, **3**, and **4**). This demonstrated that the combination of azoles and bromperidol derivatives showed better results with the more resistant fungal strain *F*. Besides POS and VOR, we found that three combinations, compounds **2-4** with KTC, showed partial synergy against both fungal strains, and one combination, compound **2** with ITC, displayed partial synergy against both fungal strains. All the combinations involving FLC only displayed weak additive effect. This suggested that POS and VOR might be the best candidates for developing combination therapy with compounds **1-5**.

Table 2. FICI values of the combination of 5 azoles and compounds **1-5** against two *C. albicans* strains.

		FLC		ITC		KTC		POS		VOR	
Cpd	R	*B*	*F*	*B*	*F*	*B*	*F*	*B*	*F*	*B*	*F*
1	H	2.00	2.00	1.50	2.00	2.00	1.00	0.75	0.19	0.63	0.75
2	F	1.50	1.50	0.75	0.56	0.75	0.75	0.75	0.16	0.75	0.19
3	Cl	1.50	2.00	2.00	2.00	0.75	0.52	0.63	0.19	0.75	0.63
4	Br	2.00	2.00	1.50	2.00	0.52	0.52	1.00	0.38	1.00	0.63
5	OMe	2.00	2.00	2.00	2.00	2.00	1.00	2.00	0.13	0.75	1.25

Strains: *B* = *C. albicans* ATCC 10231, *F* = *C. albicans* ATCC 64124.
The FICI cutoff values for determining synergy are: synergistic (SYN) if FICI ≤ 0.5, additive (ADD) if 0.5 < FICI ≤ 4, antagonistic (ANT) if FICI > 4.

Indicates synergy (SYN, both drugs showed ≥2-fold reduction in MIC value).
Indicates partial synergy (pSYN, both drugs showed decrease in MIC value and one drug showed ≥2-fold reduction in MIC value).
Indicates strong additive effect (ADD*, only one drug showed ≥2-fold reduction in MIC value).
Indicates weak additive effect (ADD, neither of the drugs showed ≥2-fold reduction in MIC value).

When looking at the five synergistic combinations of POS and compounds **1-5** against strain *F*, we noticed that smaller R substituents in our compounds seemed to correlate with lower FICI values. The FICI values increased as the size of the R substituent increased from fluorine to bromine in compounds **2-4**. This may indicate that the small size of a fluorine atom as the R substituent in our compounds may be optimal for interacting with its cellular target in fungal cells, and increase in the size of the R substituent may cause loss of engagement with the target due to steric hindrance. This postulation could also be observed by looking at the overall number of combinations that show synergistic tendency (synergy or partial synergy) amongst all 25 combinations tested in the first round. Compound **2**, with a fluorine substituent, showed the

highest number of combinations (four out of five combinations) with synergistic tendencies. This number decreases as the size of the R substituent increases (from fluorine to bromine in compounds **2-4**). Compound **5**, with a methoxy substituent, showed the best synergistic effect in combination with POS with the lowest FICI value identified in this study so far. Although methoxy group is the largest R group amongst all five compounds in this study, the presence of an oxygen atom and the lowest FICI value of 0.13 might suggest either potential hydrogen bonding involved in the interaction of compound **5** with its target or methoxy group, as a strong electron donating group, increased the π–π interaction of the connected phenyl ring with the target protein/enzyme.

With POS and VOR appearing to be the best azoles to develop combinational antifungal therapy with compounds **1-5**, we further expanded our fungal collection and tested the combination of either POS or VOR and compounds **1-5** against seven additional strains in order to better assess the potential synergy against a wide variety of pathogenic fungi with distinct biological features (Table 3). Amongst these seven additional fungal strains were five extra *C. albicans* strains, one non-*albicans Candida* strain (*C. glabrata*, strain *H*), and one filamentous fungus (*A. terreus*, strain *I*). All of these fungi are resistant to POS and VOR, except for *A. terreus*, which is sensitive to both of these azoles. The checkerboard assay results for POS and VOR in combination with compounds **1-5** against strain *B* and *F* from Table 1 were also listed in Table 3 for easy comparison.

Of the ten combinations listed in Table 3, each against nine fungal strains, we found seven combinations to display synergistic interactions. Compound **2** in combination with POS or VOR showed synergy against strains *F*, *G*, and *I* or *F* and *G*, respectively. Compound **3** with either POS or VOR exhibited synergistic interactions against strain *F*. The other compounds, **1**, **4**, and **5**, all demonstrated synergistic interactions when combined with POS against strains *F*. Compounds **4** and **5** also were found to be synergistic with POS against strains *H* and *I*, respectively. Furthermore, all ten combinations exhibited partial synergy against at least one fungal strain, four of which also displayed strong additive effect against a variety of fungal strains (compound **1** with POS or VOR, compound **3** with POS, and compound **5** with VOR). The best combination appeared to be compound **2** and POS, as this combination exhibited

synergistic interactions against three fungal strains and partial synergy against four more out of the nine fungal strains we tested. No combinations were discovered to have antagonistic interactions (FICI > 4). Additionally, we found that more combinations involving POS showed synergistic or partially synergistic effect compared to the combinations involving VOR. For instance, all five combinations involving POS displayed synergy, whereas only two out of five combinations involving VOR tested exhibited synergistic interactions. These data demonstrated better synergy from developing combination antifungal therapy with POS and bromperidol series derivatives.

Table 3. Combinational effect of POS or VOR with compounds **1-5** against a variety of fungal strains.

Cpd	Azole	Strain	MIC alone (μg/mL)		MIC combo (μg/mL)		FICI	Interp.	Azole	Strain	MIC alone (μg/mL)		MIC combo (μg/mL)		FICI	Interp.
			Azole	Cpd	Azole	Cpd					Azole	Cpd	Azole	Cpd		
1	POS	*A*	>32	>64	1	64	1.03	ADD*	VOR	*A*	>32	>64	2	64	1.06	ADD*
		B	1	>64	0.5	16	0.75	pSYN		*B*	1	>64	0.5	8	0.63	pSYN
		C	>32	>64	>32	>64	2.00	ADD		*C*	>32	>64	>32	>64	2.00	ADD
		D	>32	>64	0.25	64	1.01	ADD*		*D*	>32	>64	>32	>64	2.00	ADD
		E	>32	>64	>32	>64	2.00	ADD		*E*	>32	>64	>32	>64	2.00	ADD
		F	>32	>64	2	8	0.19	SYN		*F*	>32	>64	8	32	0.75	pSYN
		G	>32	>64	0.125	64	1.00	ADD*		*G*	>32	>64	0.125	64	1.00	ADD*
		H	>32	>64	2	32	0.56	pSYN		*H*	>32	>64	0.25	64	1.01	ADD*
		I	1	>64	0.25	64	1.25	ADD*		*I*	0.5	>64	0.5	>64	2.00	ADD
2	POS	*A*	>32	>128	8	64	0.75	pSYN	VOR	*A*	>32	>128	0.5	64	0.52	pSYN
		B	0.5	128	0.125	64	0.75	pSYN		*B*	0.5	128	0.25	32	0.75	pSYN
		C	>32	>128	>32	>128	2.00	ADD		*C*	>32	>128	>32	>128	2.00	ADD
		D	>32	>128	0.5	64	0.52	pSYN		*D*	>32	>128	1	64	0.53	pSYN
		E	>32	>128	>32	>128	2.00	ADD		*E*	>32	>128	>32	>128	2.00	ADD
		F	>32	128	4	4	0.16	SYN		*F*	>32	128	4	8	0.19	SYN
		G	>32	>128	0.0625	64	0.50	SYN		*G*	>32	>128	0.125	64	0.50	SYN
		H	>32	>128	0.25	64	0.51	pSYN		*H*	>32	>128	0.25	64	0.51	pSYN
		I	1	>128	0.25	32	0.5	SYN		*I*	0.5	>128	0.5	>128	2.00	ADD
3	POS	*A*	>32	>32	1	16	0.53	pSYN	VOR	*A*	>32	>32	8	16	0.75	pSYN
		B	1	>32	0.5	4	0.63	pSYN		*B*	1	>32	0.25	16	0.75	pSYN
		C	>32	>32	0.5	32	1.02	ADD*		*C*	>32	>32	16	16	1.00	ADD
		D	>32	>32	2	16	0.56	pSYN		*D*	>32	>32	16	16	1.00	ADD
		E	>32	>32	0.5	16	0.52	pSYN		*E*	>32	>32	16	16	1.00	ADD
		F	>32	>32	2	4	0.19	SYN		*F*	>32	>32	4	16	0.63	pSYN
		G	>32	>32	0.5	16	0.52	pSYN		*G*	>32	>32	2	8	0.31	SYN
		H	>32	>32	>32	>32	2.00	ADD		*H*	>32	>32	>32	>32	2.00	ADD
		I	0.5	>32	0.5	>32	2.00	ADD		*I*	0.25	>32	0.25	>32	2.00	ADD
4	POS	*A*	>32	>64	>32	>64	2.00	ADD	VOR	*A*	>32	>64	>32	>64	2.00	ADD
		B	1	>64	0.5	32	1.00	ADD		*B*	1	>64	0.5	32	1.00	ADD
		C	>32	>64	>32	>64	2.00	ADD		*C*	>32	>64	>32	>64	2.00	ADD
		D	>32	>64	>32	>64	2.00	ADD		*D*	>32	>64	>32	>64	2.00	ADD
		E	>32	>64	>32	>64	2.00	ADD		*E*	>32	>64	>32	>64	2.00	ADD
		F	>32	>64	4	16	0.38	SYN		*F*	>32	>64	4	32	0.63	pSYN
		G	>32	>64	>32	>64	2.00	ADD		*G*	>32	>64	>32	>64	2.00	ADD
		H	>32	>64	8	8	0.38	SYN		*H*	>32	>64	>32	>64	2.00	ADD
		I	0.5	>64	0.25	1	0.52	pSYN		*I*	0.25	>64	0.25	>64	2.00	ADD
5	POS	*A*	>32	>128	>32	>128	2.00	ADD	VOR	*A*	>32	>128	>32	>128	2.00	ADD
		B	1	>128	1	>128	2.00	ADD		*B*	1	>128	0.5	32	0.75	pSYN
		C	>32	>128	>32	>128	2.00	ADD		*C*	>32	>128	>32	>128	2.00	ADD
		D	>32	>128	>32	>128	2.00	ADD		*D*	>32	>128	>32	>128	2.00	ADD
		E	>32	>128	>32	>128	2.00	ADD		*E*	>32	>128	>32	>128	2.00	ADD
		F	>32	>128	2	8	0.13	SYN		*F*	>32	>128	8	128	1.25	ADD*
		G	>32	>128	>32	>128	2.00	ADD		*G*	>32	>128	>32	>128	2.00	ADD
		H	>32	>128	0.25	64	0.51	pSYN		*H*	>32	>128	>32	>128	2.00	ADD
		I	0.5	>128	0.125	32	0.50	SYN		*I*	0.25	>128	0.25	>128	2.00	ADD

Strains: *A* = *C. albicans* ATCC MYA-1003, *B* = *C. albicans* ATCC 10231, *C* = *C. albicans* ATCC MYA-1237, *D* = *C. albicans* ATCC MYA-2310, *E* = *C. albicans* ATCC MYA-2876, *F* = *C. albicans* ATCC 64124, *G* = *C. albicans* ATCC 90819, *H* = *C. glabrata* ATCC 2001, *I* = *A. terreus* ATCC MYA-3633.
The FICI cutoff values for determining synergy are: synergistic (SYN) if FICI ≤ 0.5, additive (ADD) if 0.5 < FICI ≤ 4, antagonistic (ANT) if FICI > 4.
Note: Where the highest concentration of a compound or azole drug alone did not achieve optical growth inhibition, the MIC_{alone} value used in the FICI calculation is the highest concentration tested of that compound or azole drug.
Indicates synergy (SYN, both drugs showed ≥2-fold reduction in MIC value).
Indicates partial synergy (pSYN, both drugs showed decrease in MIC value and drug showed ≥2-fold reduction in MIC value).
Indicates strong additive effect (ADD*, only one drug showed ≥2-fold reduction in MIC value).
Indicates weak additive effect (ADD, neither of the drugs showed ≥2-fold reduction in MIC value).

In addition to the various *C. albicans* strains, we also observed synergy in the non-*albicans Candida*, *C. glabrata*, and the filamentous fungus, *A. terreus*. Amongst the ten combinations against *C. glabrata* (strain *H*), we found one combination (compound **4** with POS) to display strong synergy, four combinations (compounds **1**, **2**, and **5** with POS as well as compound **2** with VOR) to display partial synergy, and one combination (compound **1** with VOR) to display strong additive effect. Amongst the ten combinations against *A. terreus* (strain *I*), we found two combinations (compounds **2** or **5** with POS) to be synergistic, one combination to display partial synergy, and one combination to display strong additive effect. Overall, these data further suggested that combining azoles and compounds **1**-**5** as an antifungal strategy would be effective against a variety of fungal pathogens and benefit patients suffering from different fungal infections.

Time-dependent antifungal activity of the combination of azole antifungals and bromperidol series compounds

In order to confirm the synergistic interaction between our bromperidol series compounds and azole antifungals, we performed time-kill assays against strain *F* for two representative combinations (compound **2** and either POS or VOR) that displayed great synergy in previous checkerboard assays (Fig. 2). In these time-kill assays, we evaluated the antifungal effect of each drug alone (POS, VOR, or compound **2**) as well as the two combinations (POS or VOR with compound **2**) at various concentrations (0.5×-8× MIC_{combo} of each drug). Overall, the combinations of POS or VOR and compound **2** showed fungistatic effect at 8× MIC_{combo} concentrations. The CFU/mL values did not differentiate amongst various samples until after 6 h. In the first combination tested (POS + compound **2**), we saw significant growth in the growth control (increase in CFU/mL by 4 order of magnitude). Meanwhile, the POS or compound **2** alone samples showed slight inhibition of fungal growth similar to that of the 0.5× and 1×

MIC_{combo} samples (increase in CFU/mL by about 1 order of magnitude). The combination sample with 4× MIC_{combo} showed stronger inhibition of fungal growth compared to the alone samples as well as the combination samples with less drugs. The combination sample with 8× MIC_{combo} showed complete inhibition of fungal growth and the CFU/mL value for this sample remained around 1×10^5 CFU/mL throughout 24 h. The growth of the fungus in each sample was further assessed by the addition of resazurin after the 24-h time point, which can be metabolized by live fungal cells and turns the solution from blue to pink. The combination of VOR and compound **2** showed a similar profile to that of the combination of POS and compound **2**, except that the combination sample with 8× MIC_{combo} showed slight reduction in CFU/mL by about 1 $\log_{10}$ unit over 24 h.

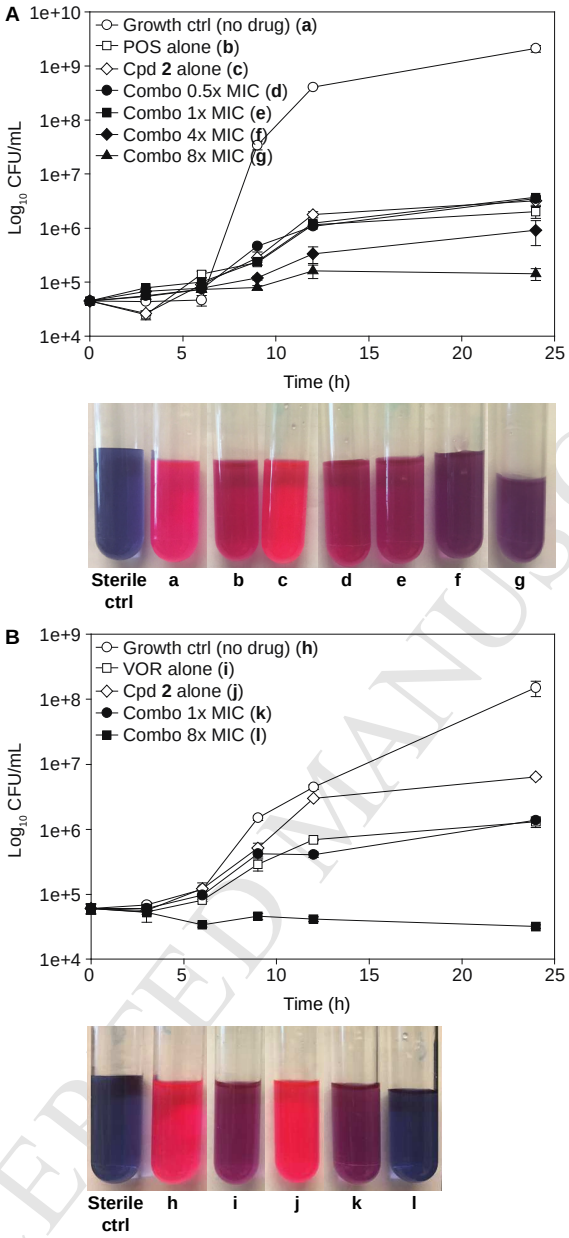

Fig. 2. Time- and dose-dependent antifungal synergy of the combination of **A.** POS or **B.** VOR and compound **2** at various concentrations against *C. albicans* ATCC 64124 (strain *F*). Each sample with resazurin added for visualization of fungal growth is presented underneath the growth curve for each combination.

Biofilm disruption with the combination of representative azole antifungals and bromperidol series compounds

Recent discoveries suggest that fungi in biofilms display drastically different biology and antimicrobial susceptibility when compared to free living (planktonic) fungal cells [30]. Biofilm-forming (sessile) fungal cells are protected by extracellular matrices and display increased

resistance against a variety of antifungal agents [31-34]. Using a water soluble metabolic dye, XTT (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide), the metabolic activity of biofilm (sessile cells) can be measured in checkerboard format assays [35]. In this study, we selected two representative combinations (POS or VOR with compound **2**), which showed excellent synergy in the above checkerboard assay against strain *F*, and evaluated the antifungal effect that these combinations have on sessile cells (Table 4). We reported that the sessile cells of strain *F* are highly resistant to all drugs tested, including POS, VOR, and compound **2** alone with sessile MIC (SMIC) values of >32, >32, and >128 μg/mL, respectively. When tested in combination, both combinations failed to show strong synergy as observed in checkerboard assay in planktonic fungal cells. Instead, both combinations showed strong additive antifungal effect with FICI values of 1.02 (the SMIC value of compound **2** in combination did not decrease by much compared to that alone, the SMIC values of POS or VOR both decreased significantly from >32 to 0.5 μg/mL). This result suggested great antifungal potential of the combination of azoles and bromperidol series compounds as well as the more resistant nature of the sessile fungal cells compared to planktonic cells.

Table 4. Inhibition of biofilm formation with azoles and compound **2** combinations.

Cpd	Azole	Strain	SMIC alone (μg/mL)		SMIC combo (μg/mL)		FICI	Interp.
			Azole	Cpd	Azole	Cpd		
2	POS	*F*	>32	>128	0.5	128	1.02	ADD*
	VOR	*F*	>32	>128	0.5	128	1.02	ADD*

Strain *F* = *C. albicans* ATCC 64124.
The FICI cutoff values for determining synergy are: synergistic (SYN) if FICI ≤ 0.5, additive (ADD) if 0.5 < FICI ≤ 4, antagonistic (ANT) if FICI > 4.
Since the highest concentration of compound **2** or azole alone did not achieve complete growth inhibition, the MIC_{alone} value used in the FICI calculation is the highest concentration tested of compound **2** or azole drugs.
ADD* indicates strong additive effect (one drug showed ≥2-fold reduction in MIC value).

Mammalian cytotoxicity of the combination of representative azole antifungals and bromperidol series compounds

In addition to assessing the time-dependent killing and disruption of fungal biofilm, we also evaluated the mammalian cytotoxicity of azole antifungals and bromperidol (**2**) alone and in combination (Fig. 3). In order to gain a better understanding of the toxicity profile towards different mammalian cells, we evaluated representative azoles (POS and VOR) and compound **2** against three different mammalian cell lines, including human bronchial epithelial cells BEAS-2B, human kidney epithelial cells HEK-293, and the murine macrophage J774A.1. Please note that as many xenobiotics stimulate cell growth instead of exerting toxicity at sub-IC_{50}

concentrations [23, 36-39], resulting in >100% cell survival in the treatment groups, we have considered these >100% cell survival data as no observed toxicity and expressed them as 100% cell survival. When testing the azole antifungals alone (Fig. 3A), we observed no cytotoxic effect up to 4 μg/mL. At 8 μg/mL, we observed 80 ± 9% cell survival with J774A.1 cells, and at 16 μg/mL, we observed around 45 ± 12% and 46 ± 3% cell survival POS-treated HEK-293 and J774A.1 cells, respectively. No cytotoxic effect was observed in any VOR-treated cell lines at any concentrations tested. With BEAS-2B and HEK-293 cells being more robust cell lines than J774A.1, we were not surprised to see the absence of toxicity in these two cell lines compared to that in J774A.1 cells, because macrophages are often short-lived and fragile within the human body. We then assessed the toxicity of compound **2** alone as a representative of our bromperidol series derivatives (Fig. 2B). We found no toxicity against BEAS-2B and HEK-293 cells at 64 μg/mL. Compound **2** alone exerted toxicity against J774A.1 cells and showed 72 ± 14%, 50 ± 7% and 30 ± 4% cell survival with 16, 32, and 64 μg/mL compound **2**, respectively. These findings suggested that our bromperidol series compounds had much better toxicity profiles against various mammalian cells and that the strategy of using our bromperidol series compounds in combination with azole antifungals can effectively help alleviate azole-induced toxicity by reducing the amount of azole antifungals required in treatment.

Since 8 μg/mL compound **2** was the highest concentration at which no cytotoxicity was observed with all three cell lines, we performed the cytotoxicity assay of POS and VOR against all three cell lines with 8 μg/mL compound **2** supplemented in the media (Fig. 3C). We observed similar overall cytotoxic effect as in the azoles alone samples. The percent cell survivals were slightly lower at 8 or 16 μg/mL POS in combination compared to that of POS alone. Meanwhile, we observed no toxicity at 16 μg/mL VOR in combination with 8 μg/mL compound **2**. As J774A.1 is the most fragile cell line tested, we also tested the combination of azoles and a higher concentration of compound **2** (32 μg/mL) against the two epithelial cell lines (BEAS-2B and HEK-293 cells) (Fig. 3D). Of the combination of POS and compound **2** against BEAS-2B cells, we observed 71 ± 11% and 38 ± 6% cell survival at 8 and 16 μg/mL POS. However, the combination toxicity of POS and 32 μg/mL compound **2** was more prominent against HEK-293 cells where we observed decreased cell survival from 80 ± 8% to 40 ± 6% as the concentration of

POS increased from 1 to 16 μg/mL. With 32 μg/mL compound **2**, we still noted no toxicity against either cell lines at any concentration of VOR, which proved the better toxicity profile of VOR compared to that of POS.

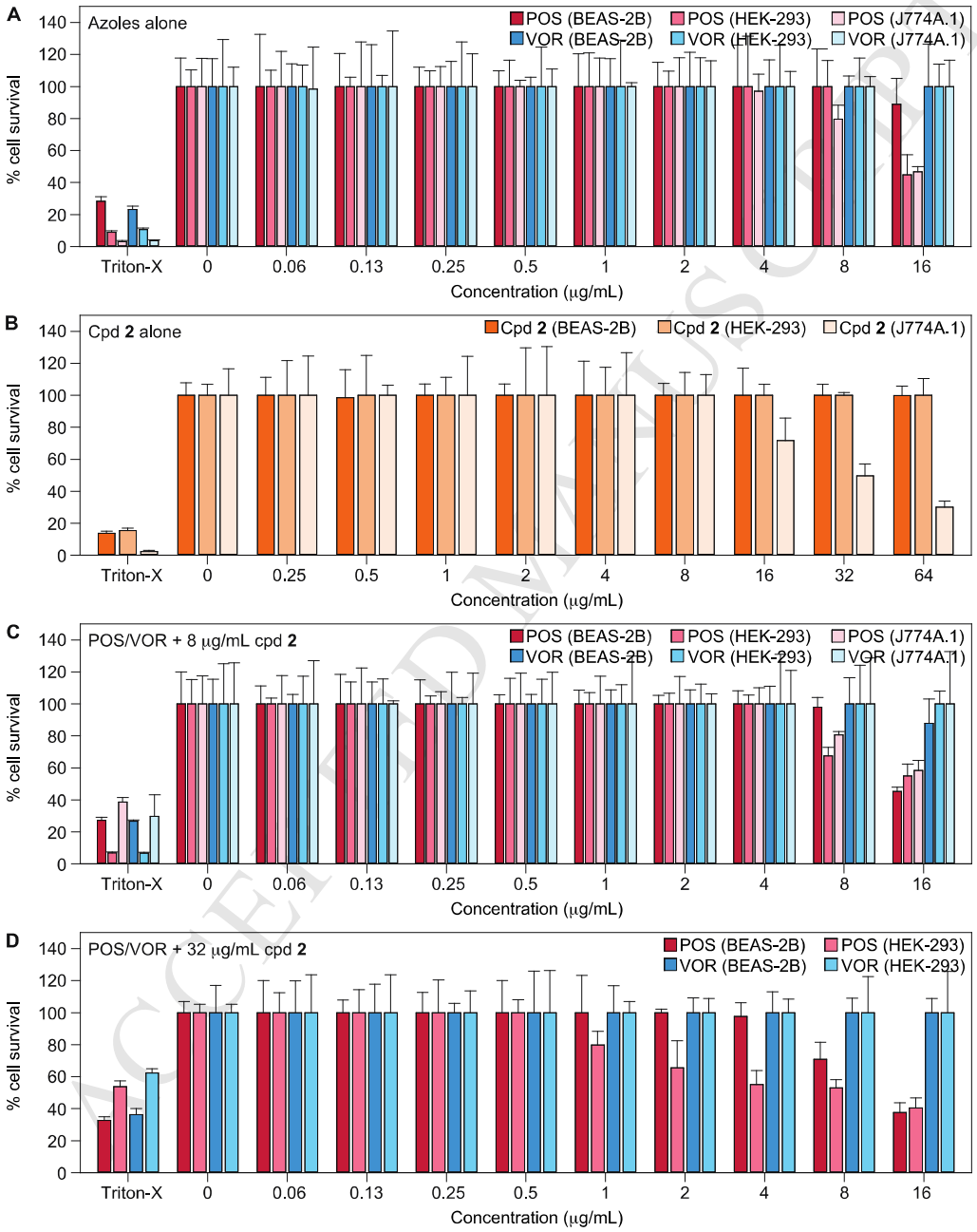

Fig. 3. Mammalian cytotoxicity evaluation of **A.** POS and VOR alone, **B.** compound **2** alone, **C.** and **D.** representative combinations of azoles (POS or VOR) at various concentrations along with compound **2** at 8 μg/mL (panel **C**) or 32 μg/mL (panel **D**) supplemented in the media against BEAS-2B, HEK-293, and J774A.1 cells. *Note*:

As at 32 μg/mL, compound **2** exerted toxicity against J774A.1 (as seen in panel **B**), this cell line was not tested in panel **D**.

Judging from the results from cytotoxicity assays, POS seemed to be toxic to mammalian cells. Thus, developing combinational antifungal therapy that involves less POS and more of the nontoxic bromperidol (**2**) seemed to be a reasonable approach to alleviate azole-induced toxicity and other related side effects. In addition to the great potential of synergy between POS and bromperidol compounds, VOR also has great potential to be developed into combinational antifungal therapies due to its nontoxic nature.

Haloperidol/bromperidol, originally antipsychotic drugs, act on dopamine D2 receptors, which is a G protein-coupled receptor with P-glycoprotein properties [40, 41]. However, as newly discovered antifungal candidates, their cellular target in fungal cells remained elusive. Although some reports indicated that haloperidol might target the biosynthesis and metabolism of amino acids [42] or fungal morphogenesis and hyphal formation [27, 43] in fungal pathogens, others pointed out that the multidrug-resistant transporter (MDR1), a p-glycoprotein, is more likely to be the antifungal target of this antipsychotic drug [44]. Inhibition of MDR1, an active transporter/efflux pump that contributes to efflux-related azole resistance, can further sensitize fungal pathogens to azole antifungals and prolong their antifungal effect. The bromperidol series compounds presented in this study, due to structural similarity, are also likely to exert their antifungal properties in the same way. Although determining the exact mechanism of action of bromperidol is outside of the scope of the current study, we wanted to offer a potential explanation for the synergy observed herein. This theory could also explain why the bromperidol series compounds possessed no antifungal activity by themselves, but could produce great antifungal synergy in combination with various azoles. A recent study reported synergistic antifungal effect of FLC and VOR in combination with haloperidol as an MDR1 inhibitor against two *Malassezia* strains [45], which also demonstrated the feasibility and benefits of developing new antifungal therapies with the combination of haloperidol or its derivatives and azole antifungals.

CONCLUSIONS

In this study, we evaluated the antifungal effect of bromperidol and four of its derivatives in combination with five clinically relevant azole antifungals against a wide variety of pathogenic fungi. From our extensive evaluation of the combinational antifungal effect between the two classes of compounds by checkerboard, time-kill, and biofilm disruption assays, we observed a wide range of combinational effects ranging from synergistic to weak additive effect. A considerable portion of the combinations tested in this study displayed synergy or partial synergy. We also found that POS displayed synergy in more combinations with bromperidol series compounds than VOR did. However, our cytotoxicity evaluation suggested combination therapy with VOR might have superior mammalian cytotoxicity profiles. As mentioned above, the FICI calculated in this study are likely to be higher than the true FICI values due to the unbound MIC values. Therefore, the potential synergy and the number of combinations showing synergistic effects are also likely to be underestimated. Even though the exact cellular target by which the bromperidol series compounds exert antifungal activity when combined with azole antifungals remains unclear, our results suggested that using these bromperidol derivatives in combination with clinically relevant azoles can synergistically inhibit fungal growth and effectively reduced the amount of azoles required to achieve an equivalent antifungal effect, and therefore, alleviate the toxicity and side effects resulted from administering high concentrations of azole antifungals.

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CONFLICTS OF INTEREST

None.

CONTRIBUTORS

S.Y.L.H. and S.G.-T. were involved in the study design presented in this manuscript. A.G. carried out the design and synthesis of compounds **1-5**. All biological evaluations were performed by S.Y.L.H. (checkerboard, time-kill, and cytotoxicity assays), E.K.D. (checkerboard

assays), and S.K.S. (biofilm disruption assays). The manuscript and all associated documents were produced by S.Y.L.H. and S.G.-T. with the assistance of all other co-authors listed.

REFERENCES

[1] H.X. Ngo, S. Garneau-Tsodikova, K.D. Green, A complex game of hide and seek: the search for new antifungals, MedChemComm, 7 (2016) 1285-1306.

[2] M.C. Arendrup, Epidemiology of invasive candidiasis, Curr. Opin. Crit. Care, 16 (2010) 445-452.

[3] P. Vandeputte, S. Ferrari, A.T. Coste, Antifungal resistance and new strategies to control fungal infections, Int. J. Microbiol., 2012 (2012) 713687.

[4] M.A. Pfaller, D.J. Diekema, The Epidemiology of invasive candidiasis, in: R.A. Calderone, C.J. Clancy (Eds.) *Candida* and candidiasis, ASM Press, Washington, DC, 2012, pp. 449-450.

[5] D.W. Denning, A. Pleuvry, D.C. Cole, Global burden of allergic bronchopulmonary aspergillosis with asthma and its complication chronic pulmonary aspergillosis in adults, Med. Mycol., 51 (2013) 361-370.

[6] P.E. Russell, A century of fungicide evolution, J. Agr. Sci., 143 (2005) 11-25.

[7] K. Yoshida, V.J. Schuenemann, L.M. Cano, M. Pais, B. Mishra, R. Sharma, C. Lanz, F.N. Martin, S. Kamoun, J. Krause, M. Thines, D. Weigel, H.A. Burbano, The rise and fall of the *Phytophthora infestans* lineage that triggered the Irish potato famine, Elife, 2 (2013) e00731.

[8] R.P. Singh, D.P. Hodson, J. Huerta-Espino, Y. Jin, S. Bhavani, P. Njau, S. Herrera-Foessel, P.K. Singh, S. Singh, V. Govindan, The emergence of Ug99 races of the stem rust fungus is a threat to world wheat production, Annu. Rev. Phytopathol., 49 (2011) 465-481.

[9] P.G. Pappas, C.A. Kauffman, D.R. Andes, C.J. Clancy, K.A. Marr, L. Ostrosky-Zeichner, A.C. Reboli, M.G. Schuster, J.A. Vazquez, T.J. Walsh, T.E. Zaoutis, J.D. Sobel, Clinical practice guideline for the management of candidiasis: 2016 update by the infectious diseases society of America, Clin. Infect. Dis., 62 (2016) e1-e50.

[10] R. Laniado-Laborin, M.N. Cabrales-Vargas, Amphotericin B: side effects and toxicity, Rev. Iberoam. Micol., 26 (2009) 223-227.

[11] J.H. Rex, J.E. Bennett, A.M. Sugar, P.G. Pappas, C.M. van der Horst, J.E. Edwards, R.G. Washburn, W.M. Scheld, A.W. Karchmer, A.P. Dine, et al., A randomized trial comparing fluconazole with amphotericin B for the treatment of candidemia in patients without neutropenia. Candidemia Study Group and the National Institute, N. Engl. J. Med., 331 (1994) 1325-1330.

[12] C. Charlier, E. Hart, A. Lefort, P. Ribaud, F. Dromer, D.W. Denning, O. Lortholary, Fluconazole for the management of invasive candidiasis: where do we stand after 15 years?, J. Antimicrob. Chemother., 57 (2006) 384-410.

[13] M.A. Ghannoum, L.B. Rice, Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance, Clin. Microbiol. Rev., 12 (1999) 501-517.

[14] D.J. Sheehan, C.A. Hitchcock, C.M. Sibley, Current and emerging azole antifungal agents, Clin. Microbiol. Rev., 12 (1999) 40-79.

[15] V.J. Lempers, L.C. Martial, M.F. Schreuder, N.M. Blijlevens, D.M. Burger, R.E. Aarnoutse, R.J. Bruggemann, Drug-interactions of azole antifungals with selected immunosuppressants in transplant patients: strategies for optimal management in clinical practice, Curr. Opin. Pharmacol., 24 (2015) 38-44.

[16] R. Prasad, A.H. Shah, M.K. Rawal, Antifungals: Mechanism of Action and Drug Resistance, Adv. Exp. Med. Biol., 892 (2016) 327-349.

[17] S.K. Shrestha, A. Garzan, S. Garneau-Tsodikova, Novel alkylated azoles as potent antifungals, Eur. J. Med. Chem., 133 (2017) 309-318.

[18] V. Nagappan, S. Deresinski, Reviews of anti-infective agents: posaconazole: a broad-spectrum triazole antifungal agent, Clin. Infect. Dis., 45 (2007) 1610-1617.

[19] L. Xiao, V. Madison, A.S. Chau, D. Loebenberg, R.E. Palermo, P.M. McNicholas, Three-dimensional models of wild-type and mutated forms of cytochrome P450 14alpha-sterol demethylases from *Aspergillus fumigatus* and *Candida albicans* provide insights into posaconazole binding, Antimicrob. Agents Chemother., 48 (2004) 568-574.

[20] L.E. Cowen, S.D. Singh, J.R. Kohler, C. Collins, A.K. Zaas, W.A. Schell, H. Aziz, E. Mylonakis, J.R. Perfect, L. Whitesell, S. Lindquist, Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease, Proc. Natl. Acad. Sci., U. S. A., 106 (2009) 2818-2823.

[21] R. Mangoyi, J. Midiwo, S. Mukanganyama, Isolation and characterization of an antifungal compound 5-hydroxy-7,4'-dimethoxyflavone from *Combretum zeyheri*, BMC Complement Altern. Med., 15 (2015) 405.

[22] K. Niimi, D.R. Harding, R. Parshot, A. King, D.J. Lun, A. Decottignies, M. Niimi, S. Lin, R.D. Cannon, A. Goffeau, B.C. Monk, Chemosensitization of fluconazole resistance in Saccharomyces cerevisiae and pathogenic fungi by a D-octapeptide derivative, Antimicrob. Agents Chemother., 48 (2004) 1256-1271.

[23] 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.

[24] S.K. Shrestha, M.Y. Fosso, S. Garneau-Tsodikova, A combination approach to treating fungal infections, Sci. Rep., 5 (2015) 17070.

[25] B. Zhai, C. Wu, L. Wang, M.S. Sachs, X. Lin, The antidepressant sertraline provides a promising therapeutic option for neurotropic cryptococcal infections, Antimicrob. Agents Chemother., 56 (2012) 3758-3766.

[26] J. Afeltra, P.E. Verweij, Antifungal activity of nonantifungal drugs, Eur. J. Clin. Microbiol. Infect. Dis., 22 (2003) 397-407.

[27] M. Stylianou, E. Kulesskiy, J.P. Lopes, M. Granlund, K. Wennerberg, C.F. Urban, Antifungal application of nonantifungal drugs, Antimicrob. Agents Chemother., 58 (2014) 1055-1062.

[28] S. Ramon-Garcia, C. Ng, H. Anderson, J.D. Chao, X. Zheng, T. Pfeifer, Y. Av-Gay, M. Roberge, C.J. Thompson, Synergistic drug combinations for tuberculosis therapy identified by a novel high-throughput screen, Antimicrob. Agents Chemother., 55 (2011) 3861-3869.

[29] J. Meletiadis, J.W. Mouton, J.F. Meis, P.E. Verweij, *In vitro* drug interaction modeling of combinations of azoles with terbinafine against clinical *Scedosporium prolificans* isolates, Antimicrob. Agents Chemother., 47 (2003) 106-117.

[30] R.M. Donlan, Biofilms: microbial life on surfaces, Emerg. Infect. Dis., 8 (2002) 881-890.

[31] J.W. Costerton, P.S. Stewart, E.P. Greenberg, Bacterial biofilms: a common cause of persistent infections, Science, 284 (1999) 1318-1322.

[32] G. Ramage, S.P. Saville, D.P. Thomas, J.L. Lopez-Ribot, *Candida* biofilms: an update, Eukaryot. Cell, 4 (2005) 633-638.

[33] S.P. Bachmann, K. VandeWalle, G. Ramage, T.F. Patterson, B.L. Wickes, J.R. Graybill, J.L. Lopez-Ribot, *In vitro* activity of caspofungin against *Candida albicans* biofilms, Antimicrob. Agents Chemother., 46 (2002) 3591-3596.

[34] M.A. Al-Fattani, L.J. Douglas, Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance, J. Med. Microbiol., 55 (2006) 999-1008.

[35] C.G. Pierce, P. Uppuluri, A.R. Tristan, F.L. Wormley, Jr., E. Mowat, G. Ramage, J.L. Lopez-Ribot, A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing, Nat. Protoc., 3 (2008) 1494-1500.

[36] C.L. Quave, M. Estevez-Carmona, C.M. Compadre, G. Hobby, H. Hendrickson, K.E. Beenken, M.S. Smeltzer, Ellagic acid derivatives from *Rubus ulmifolius* inhibit *Staphylococcus aureus* biofilm formation and improve response to antibiotics, PLoS One, 7 (2012) e28737.

[37] B.S. Hall, C. Bot, S.R. Wilkinson, Nifurtimox activation by trypanosomal type I nitroreductases generates cytotoxic nitrile metabolites, J. Biol. Chem., 286 (2011) 13088-13095.

[38] W. Xu, X. Zhu, T. Tan, W. Li, A. Shan, Design of embedded-hybrid antimicrobial peptides with enhanced cell selectivity and anti-biofilm activity, PLoS One, 9 (2014) e98935.

[39] 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.

[40] P. Seeman, Atypical antipsychotics: mechanism of action, Can. J. Psychiatry, 47 (2002) 29-40.

[41] D.E. Grigoriadis, H.B. Niznik, K.R. Jarvie, P. Seeman, Glycoprotein nature of D2 dopamine receptors, FEBS Lett., 227 (1988) 220-224.

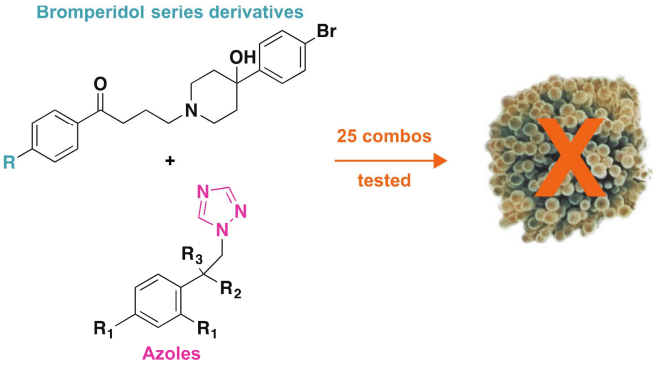
[42] E. Ericson, M. Gebbia, L.E. Heisler, J. Wildenhain, M. Tyers, G. Giaever, C. Nislow, Off-target effects of psychoactive drugs revealed by genome-wide assays in yeast, PLoS Genet., 4 (2008) e1000151.

[43] T. Miwa, Y. Takagi, M. Shinozaki, C.W. Yun, W.A. Schell, J.R. Perfect, H. Kumagai, H. Tamaki, Gpr1, a putative G-protein-coupled receptor, regulates morphogenesis and hypha formation in the pathogenic fungus *Candida albicans*, Eukaryot. Cell, 3 (2004) 919-931.

[44] K. Iwaki, T. Sakaeda, M. Kakumoto, T. Nakamura, C. Komoto, N. Okamura, K. Nishiguchi, T. Shiraki, M. Horinouchi, K. Okumura, Haloperidol is an inhibitor but not substrate for MDR1/P-glycoprotein, J. Pharm. Pharmacol., 58 (2006) 1617-1622.

[45] R. Iatta, M.R. Puttilli, D. Immediato, D. Otranto, C. Cafarchia, The role of drug efflux pumps in Malassezia pachydermatis and *Malassezia furfur* defence against azoles, Mycoses, 60 (2017) 178-182.

Image for Table of Content



Highlights:

- Azole antifungals and bromperidol series derivatives show antifungal synergy.
- Posaconazole shows synergistic interaction with most bromperidol compounds.
- Combination of azoles and bromperidol compounds show fungistatic effect.
- Azole and bromperidol combinations disrupt fungal biofilm with additive effect.
- Combination of azoles and bromperidol compounds can be a new antifungal strategy.