Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Enhanced activity of antifungal drugs using natural phenolics against yeast strains of *Candida* and *Cryptococcus*

N.C.G.Faria[1], J.H. Kim[3], L.A.P. Gonçalves[2], M. de L. Martins[1], K.L. Chan[3] and B.C. Campbell[3]

1 Instituto de Higiene e Medicina Tropical/CREM, Universidade Nova de Lisboa, Portugal
2 Instituto de Higiene e Medicina Tropical/CEAUL, Universidade Nova de Lisboa, Portugal
3 Plant Mycotoxin Research Unit, Western Regional Research Center, ARS-USDA, Albany, CA, USA

Keywords
amphotericin B, phenolic, synergism, triazole.

Correspondence
Bruce C. Campbell, Plant Mycotoxin Research Unit, Western Regional Research Center, USDA-ARS, 800 Buchanan Street, Albany, CA 94710, USA.
E-mail: bruce.campbell@ars.usda.gov

2010/1438: received 19 August 2010, revised 14 February 2011 and accepted 15 February 2011

doi:10.1111/j.1472-765X.2011.03032.x

Abstract

Aims: Determine whether certain, natural phenolic compounds enhance activity of commercial antifungal drugs against yeast strains of *Candida* and *Cryptococcus neoformans*.

Methods and Results: Twelve natural phenolics were examined for fungicidal activity against nine reference strains of *Candida* and one of *C. neoformans*. Six compounds were selected for synergistic enhancement of antifungal drugs, amphotericin B (AMB), fluconazole (FLU) and itraconazole (ITR). Matrix assays of phenolic and drug combinations conducted against one reference strain, each, of *Candida albicans* and *C. neoformans* showed cinnamic and benzoic acids, thymol, and 2,3- and 2,5-dihydroxybenzaldehydes (-DBA) had synergistic interactions depending upon drug and yeast strain. 2,5-DBA was synergistic with almost all drug and strain combinations. Thymol was synergistic with all drugs against *Ca. albicans* and with AMB in *C. neoformans*. Combinations of benzoic acid or thymol with ITR showed highest synergistic activity. Of 36 combinations of natural product and drug tested, none were antagonistic.

Conclusions: Relatively nontoxic natural products can synergistically enhance antifungal drug activity, *in vitro*.

Significance and Impact of the Study: This is a proof-of-concept, having clinical implications. Natural chemosensitizing agents could lower dosages needed for effective chemotherapy of invasive mycoses. Further studies against clinical yeast strains and use of animal models are warranted.

Introduction

Resistance to antifungal agents is widely recognized (Denning *et al.* 1997; Pfaller *et al.* 2006) requiring continuous development of new antifungal agents (Stevens *et al.* 2004). Candidiasis and cryptococcosis of continuously expanding global incidence are a result of increased immunosuppressive disorders, including AIDS and certain chemo- or radiotherapies (Sobel *et al.* 2004).

Commonly prescribed drugs for the treatment of candidiases include a variety of imidazole and triazole drugs that disrupt biosynthesis of ergosterol, a fungal-specific sterol of cellular membranes (Heimark *et al.* 2002). Also included are the polyene drugs, namely amphotericin B (AMB) and nystatin, which complex with membrane sterols resulting in cellular leakage and echinochandins, which inhibit synthesis of cell wall β-(1,3)-glucans (Stevens *et al.* 2004; VandenBussche and Van Loo 2010). Treatment of cryptococcal meningitis has chiefly involved combination antifungal therapy, more than one drug, with AMB or triazoles, sometimes having severe side effects (Johnson and Perfect 2007).

Thorough investigations of fungal resistance mechanisms have involved azole antifungal agents and a wide number of *Candida* species (Pfaller *et al.* 2005). Extensive use of one antifungal drug, fluconazole (FLU), has

resulted in widespread resistance (Spanakis *et al.* 2006) associated with drug efflux through ABC plasma membrane transporters (Holmes *et al.* 2008).

There are frequent reports of cross-resistance to both FLU and itraconazole (ITR), and other azole drugs for the treatment of candidiasis and other opportunistic mycoses associated with AIDS chemotherapy (de Repentigny *et al.* 2004; Charlier *et al.* 2006). High levels of resistance to azole antifungal drugs have also been observed in other conditions involving filamentous fungi (Mocroft *et al.* 2005).

The upsurge of fungal resistance to currently available agents, and stagnation in development of new agents, compels the development of a supplementary strategy for antifungal chemotherapy. Natural compounds are a potential source of antimycotic agents either in their nascent form or as template structures for more effective derivatives (Barrett 2002; Jacob and Walker 2005). Recently, natural products were found to augment *in vitro* activity of FLU against strains of resistant filamentous fungi causing aspergillosis (Kim *et al.* 2008a, 2010). We report here the potential for safe, natural compounds to similarly improve effectiveness of selected azole and polyene antifungal drugs against the yeasts *Candida albicans* and *Cryptococcus neoformans.* These natural compounds are common phenolics found in edible plants and are considered to be innately safe for humans. This proof-of-concept, which chemosensitization by natural products enhances antifungal drug activity, warrants further testing on clinical strains and in animal models.

Materials and methods

Antifungal drugs and chemicals

All benzo analogues, antifungal drugs and culture media were procured from Sigma-Aldrich, or Fluka (St Louis, MO, USA): *natural products*: cinnamic, 2-hydroxycinnamic, 3-hydroxycinnamic, 4-hydroxycinnamic, benzoic and salicylic acids, thymol, vanillin, 3,4,5-trimethoxybenzaldehyde, veratraldehyde, 2,3-dihydroxybenzaldehyde (2,3-DBA) and 2,5-dihydroxybenzaldehyde (2,5-DBA). *Antifungal drugs*: AMB, FLU, ITR. *Solvent for test compounds*: dimethylsulfoxide (DMSO).

Yeast species and strains

Candida. American Type Culture Collection (ATCC), Manassas, VA, USA – *Candida albicans* – ATCC 90028, ATCC 10231; *Candida parapsilosis* – ATCC 22019. Portuguese Yeast Culture Collection (PYCC), Universidade Nova de Lisboa – *Ca. albicans* – PYCC 3436^T, *Ca. parapsilosis* – PYCC 2545, *Candida glabrata* – PYCC 2418^T, *Candida tropicalis* – PYCC 3097^T, *Candida krusei* – PYCC 3341, *Candida lusitaniae* – PYCC 2705^T. *Cryptococcus:* Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands – *Cryptococcus neoformans* – CBS 132^T.

Antifungal activities of natural products

All reference strains of *Candida* and *C. neoformans* were used to determine antifungal activity of 12 natural products using microtitre plate assays. Natural products were dissolved in DMSO and then added to stock synthetic glucose (SG) media (6·7 mg ml^{-1} yeast nitrogen base w/o amino acids, 2% glucose) prior to distribution into microtitre plate wells. For each well, one containing only SG and DMSO (control) and another one containing also the natural product to be tested, an inoculum of *c.* 5×10^3 yeast cells (ATCC and PYCC) in SG liquid medium was added to each well containing SG and natural product to a final volume of 200 μl and final concentration of natural product to 5 mmol l^{-1}. Each test was carried out in triplicate and incubated at 30°C. Yeast cell multiplication was monitored at 48 and 72 h for *Candida* strains and *C. neoformans*, respectively, by optical density at 595 nm (OD_{595}) (Zenyth 3100 multimode detector; Anthos Labtec Instr., Salzburg, Austria). Inhibition was measured as percentage OD_{595} treated/OD_{595} control.

Determination of inhibitory concentrations

Inhibitory concentrations (IC) of antifungal drugs and natural compounds against the yeasts were determined using microdilution assays measured by optical density (Galagiani and Stevens 1978). All IC assays were performed in triplicate in microtitre plates and used an inoculum of *c.* 5×10^3 cells in SG. The final volume including SG, cells and test compound in each well was 200 μl. IC assays of natural products involved six serial, twofold dilutions using SG, starting at 5 mmol l^{-1} and ending at 78·125 μmol l^{-1}. IC determinations of antifungal drugs involved diluting FLU and ITR in DMSO, and AMB in water, and included 10 twofold serial dilutions from the starting concentration. The range of drug concentrations for calculating ICs was based on published results (Cuenca-Estrella *et al.* 2002). For the calculation of ICs for AMB, concentrations ranged from 32 to 0·03125 mg l^{-1} for strains of *Candida* and 64 to 0·0625 mg l^{-1} for *C. neoformans*. For FLU, concentrations ranged from 64 to 0·0625 mg l^{-1} for *Candida* and 128 to 0·125 mg l^{-1} for *C. neoformans*. For ITR, concentrations ranged from 4 to 0·00391 mg l^{-1} for *Candida* and 8 to 0·00781 mg l^{-1} for *C. neoformans*. Control wells received only SG and DMSO and the respective yeast-strain inoculum. Inhibition was monitored and calculated based on OD_{595} of treated *vs* control.

Matrix assays with drugs and natural products

To determine interactions of natural products and drug activities, a series of microtitre plate matrix assays were performed. For these assays, reference strains *Ca. albicans* ATCC 90028, recommended as a standard for drug resistance studies (Clinical and Laboratory Standards Institute 2007), and *C. neoformans* CBS 132^T, originally isolated from fermenting fruit juice (Guého *et al.* 1993), were used. Neither of these strains had previously been exposed to AMB, FLU or ITR. Individual drug and natural product were combined in a matrix-like fashion, in triplicate, using combinations of control, IC_{25}, IC_{50}, IC_{75} and IC_{90} levels respective to each yeast strain, using microdilution protocols described.

Statistical methods

Significant differences ($P < 0{\cdot}05$) in growth inhibition by natural products were based on the nonparametric Friedman test using SPSS (Chicago, IL, USA). IC levels corresponding to 25, 50, 75 and 90% growth inhibition (i.e. IC_{25}, IC_{50}, IC_{75} and IC_{90}) were determined by generalized linear model and probit procedures (Finney 1971) of the SPSS package. Interactions between natural compounds and antifungal drugs were determined from drug/natural product concentrations resulting in 90% growth inhibition to reduce errors in assigning interactions to 'neutral' (Meletiadis *et al.* 2005). Fractional Inhibitory Concentration Indices ($FICI_{90}$) were calculated from the lowest IC (i.e. IC_{25}, IC_{50}, IC_{75} or IC_{90}) of antifungal drug showing ≥90% growth inhibition with corresponding lowest IC_x of natural product, as follows: $FICI_{90}$ = (lowest IC_x of natural product in combination with antifungal drug resulting in ≥90% growth inhibition/IC_{90} of natural product, alone) + (lowest IC_x of antifungal drug resulting in ≥90% growth inhibition in combination with natural product/IC_{90} of antifungal drug, alone). Interactions between natural products and antifungal drugs were defined as follows: synergy = $FICI_{90} \leq 0{\cdot}5$; additive = $0{\cdot}5 < FICI_{90} \leq 1$; neutral = $1 < FICI_{90} \leq 2$; antagonistic = $2 < FICI_{90}$ (Isenberg 1992).

Results

Effects of natural products on reference strains

Of the 12 natural products tested, cinnamic, benzoic and salicylic acids, thymol, 2,3- and 2,5-DBAs (Group A) significantly inhibited growth ≥90% ($P < 0{\cdot}05$) of all strains of *Candida* and *C. neoformans* (Table 1). Although vanillin showed significant inhibitory activity against the *Candida* strains, it had only moderate antifungal activity against *C. neoformans*.

IC levels of Group A compounds varied against *Candida* strains (Table 2). For example, based on IC_{50}, fungicidal ranking, highest to lowest, was 2,3-DBA > salicylic > benzoic > cinnamic acids > thymol > 2,5-DBA. Also, among the *Candida* strains, there was a variation in sensitivities. Both *Ca. albicans* PYCC 3436^T and *Ca. lusitaniae* PYCC 2705^T were significantly more susceptible to the natural compounds, in general, than the other *Candida*, whereas *Ca. krusei* PYCC 3341and *Ca. parapsilosis* PYCC 2545 were significantly less sensitive than other *Candida*.

Table 1 Growth inhibitory activity (%) of natural products (5 mmol l^{-1}) on reference strains of *Candida* and *Cryptococcus neoformans*. Compounds having ≥90% growth inhibition ($P < 0{\cdot}05$) in all strains are in bold (Group A)

	Candida albicans			*Candida parapsilosis*		*Candida glabrata*	*Candida tropicalis*	*Candida krusei*	*Candida lusitaniae*	*C. neoformans*
Natural product	ATCC 90028	ATCC 10231	PYCC 3436^T	ATCC 22019	PYCC 2545	PYCC 2418^T	PYCC 3097^T	PYCC 3341	PYCC 2705^T	CBS 132^T
Cinnamic acid	**99·8**	**99·9**	**99·0**	**99·9**	**99·3**	**99·8**	**99·9**	**99·9**	**99·9**	**96·1**
2-Hydroxycinnamic acid	50·4	11·7	65·4	19·5	57·1	17·1	27·1	32·3	29·2	78·0
3-Hydroxycinnamic acid	63·2	44·9	80·5	32·1	41·6	8·7	39·34	19·0	40·8	81·0
4-Hydroxycinnamic acid	65·5	15·4	79·0	30·2	7·7	8·5	21·23	5·9	24·0	60·4
Veratraldehyde	32·0	16·2	87·7	32·5	54·0	10·2	15·0	34·3	18·1	88·7
Vanillin	96·3	97·8	98·8	98·0	97·8	90·5	98·7	90·2	97·9	79·3
Benzoic acid	**99·7**	**99·9**	**99·9**	**99·23**	**98·8**	**99·6**	**99·8**	**99·5**	**99·8**	**95·8**
3,4,5-Trimethoxybenzaldehyde	42·2	8·6	76·3	49·7	38·8	20·3	35·7	16·6	34·3	76·0
Salicylic acid	**99·6**	**99·9**	**99·9**	**99·8**	**98·1**	**99·9**	**99·8**	**99·9**	**99·8**	**95·4**
Thymol	**99·9**	**99·9**	**99·9**	**99·6**	**99·7**	**99·9**	**99·8**	**99·9**	**99·8**	**95·23**
2,5-DBA	**96·3**	**96·0**	**93·2**	**90·0**	**91·4**	**93·8**	**95·9**	**93·6**	**95·9**	**93·5**
2,3-DBA	**99·4**	**99·7**	**99·3**	**98·8**	**97·3**	**99·9**	**99·4**	**99·7**	**99·5**	**95·1**

DBA, dihydroxybenzaldehyde.

Table 2 Inhibitory concentrations (IC; mmol l^{-1}) of Group A natural products (see Table 1) at 25, 50, 75 and 90 levels against reference strains of *Candida* and *Cryptococcus neoformans*

	Candida albicans			*Candida parapsilosis*		*Candida glabrata*	*Candida tropicalis*	*Candida krusei*	*Candida lusitaniae*	*C. neoformans*
	ATCC 90028	ATCC 10231	PYCC 3436^T	ATCC 22019	PYCC 2545	PYCC 2418^T	PYCC 3097^T	PYCC 3341	PYCC 2705^T	CBS 132^T
Cinnamic acid										
IC_{25}	0·37	0·53	0·24	0·05	0·08	0·04	0·08	0·16	0·05	0·06
IC_{50}	0·64	0·74	0·45	0·12	0·18	0·09	0·16	0·29	0·10	0·11
IC_{75}	1·12	1·03	0·86	0·28	0·41	0·23	0·31	0·52	0·19	0·23
IC_{90}	1·86	1·38	1·53	0·62	0·88	0·55	0·57	0·88	0·33	0·44
Ave.	1·00	0·92	0·768	0·27	0·38	0·23	0·28	0·46	0·17	0·21
Benzoic acid										
IC_{25}	0·08	0·43	0·04	0·03	0·04	0·01	0·23	0·24	0·02	0·13
IC_{50}	0·19	0·73	0·10	0·09	0·13	0·05	0·48	0·71	0·06	0·26
IC_{75}	0·48	1·24	0·25	0·29	0·36	0·223	1·01	2·13	0·14	0·51
IC_{90}	1·12	2·00	0·58	0·85	0·92	0·94	1·96	7·74	0·31	0·94
Ave.	0·47	1·10	0·24	0·32	0·36	0·31	0·92	2·70	0·13	0·46
Salicylic acid										
IC_{25}	0·14	0·43	0·01	0·05	0·09	0·07	0·24	0·11	0·04	0·14
IC_{50}	0·26	0·76	0·05	0·17	0·25	0·20	0·45	0·20	0·10	0·35
IC_{75}	0·46	1·34	0·19	0·61	0·68	0·62	0·84	0·36	0·25	0·90
IC_{90}	0·78	2·23	0·70	1·96	1·66	1·72	1·47	0·61	0·58	2·12
Ave.	0·41	1·19	0·24	0·70	0·67	0·65	0·75	0·32	0·24	0·88
Thymol										
IC_{25}	0·60	0·25	0·07	0·06	0·24	0·14	0·14	0·14	0·05	0·06
IC_{50}	1·10	0·51	0·19	0·34	0·56	0·54	0·52	0·48	0·18	0·16
IC_{75}	2·02	1·02	0·49	1·97	1·28	2·06	1·95	1·72	0·66	0·46
IC_{90}	3·49	1·92	1·13	5·00*	2·72	5·00*	5·00*	5·00*	2·08	1·18
Ave.	1·80	0·92	0·47	1·84	1·20	1·97	1·90	1·86	0·74	0·46
2,5-DBA										
IC_{25}	0·71	0·35	0·02	0·21	0·13	0·35	0·99	0·20	0·14	0·02
IC_{50}	1·13	0·59	0·06	0·88	0·68	1·16	2·50	0·82	0·35	0·08
IC_{75}	1·79	1·00	0·24	3·65	3·45	3·79	5·00	3·41	0·88	0·26
IC_{90}	2·72	1·59	0·81	5·00*	5·00*	5·00*	5·00*	5·00*	2·04	0·76
Ave.	1·59	0·88	0·28	2·43	4·26	2·57	3·38	2·35	0·85	0·28
2,3-DBA										
IC_{25}	0·03	0·03	<0·01	0·03	0·05	<0·01	0·03	0·03	0·03	0·01
IC_{50}	0·04	0·05	0·01	0·08	0·09	<0·01	0·06	0·08	0·16	0·03
IC_{75}	0·07	0·07	0·02	0·18	0·18	0·01	0·10	0·14	0·78	0·12
IC_{90}	0·10	0·10	0·06	0·37	0·38	0·03	0·17	0·61	3·17	0·38
Ave.	0·058	0·06	0·02	0·16	0·18	0·01	0·09	0·21	1·04	0·13
Average: all compounds	1·94	1·92	0·40	1·60	4·20	1·40	2·21	6·32	0·59	0·40

*5 mmol l^{-1} (Table 1).
DBA, dihydroxybenzaldehyde.

ICs of natural products also varied against *C. neoformans* CBS 132^T (Table 2). Overall, the *C. neoformans* strain had an average IC level (25–90) of 0·40 mmol l^{-1}, similar to the most sensitive *Candida* strain, PYCC 3436^T (0·40 mmol l^{-1}). The hierarchy in natural product ICs differed in the *C. neoformans* strain from *Candida* strains with highest to lowest IC_{50} levels at 2,3- > 2,5-DBAs > cinnamic > thymol > benzoic > salicylic. Similar to *Candida* strains, 2,3-DBA had the highest fungicidal activity against *C. neoformans* of all natural products.

Antifungal drug activities

IC levels for 25, 50, 75 and 90% growth inhibition of AMB, FLU and ITR for *Ca. albicans* ATCC 90028 were *c.* 10^3–10^4 greater in antifungal activity than the natural products (Table 3). In general, IC_{50} levels of Group A natural products for the *Candida* ranged from 4 μmol l^{-1} (2,3-DBA, *Ca. glabrata*) to 2·5 mmol l^{-1} (2,5-DBA, *Ca. tropicalis*) (Table 2). By comparison, the highest IC_{50} for an antifungal drug was 26 μmol l^{-1} (8 mg l^{-1}, FLU, *Ca. parapsilosis*

Table 3 Inhibitory concentrations (IC; mg l^{-1}) for antifungal drugs, amphotericin B (AMB), fluconazole (FLU) and itraconazole (ITR) at 25, 50, 75 and 90 levels against reference strains of *Candida* and *Cryptococcus neoformans*

	Candida albicans			*Candida parapsilosis*		*Candida glabrata*	*Candida tropicalis*	*Candida krusei*	*Candida lusitaniae*	*C. neoformans*
	ATCC 90028	ATCC 10231	PYCC 3436^T	ATCC 22019	PYCC 2545	PYCC 2418^T	PYCC 3097^T	PYCC 3341	PYCC 2705^T	CBS 132^T
AMB										
IC_{25}	0·65	0·33	0·15	0·13	0·14	0·16	0·19	0·61	0·32	0·06
IC_{50}	1·14	0·56	0·47	0·45	0·43	0·35	0·72	1·06	0·89	0·29
IC_{75}	2·00	0·97	1·50	1·60	1·34	0·77	2·65	1·86	2·48	1·30
IC_{90}	3·30	1·58	4·27	5·02	3·71	1·57	8·59	3·09	6·17	5·11
Ave.	1·77	0·86	1·59	1·80	1·40	0·71	3·04	1·65	2·46	1·69
FLU										
IC_{25}	0·53	3·53	0·13	2·40	0·34	n/c*	0·12	1·71	n/c	0·10
IC_{50}	1·48	7·84	0·67	8·01	1·74	n/c	0·99	3·17	n/c	0·51
IC_{75}	4·11	17·39	3·43	26·77	8·98	n/c	0·82	5·89	n/c	2·75
IC_{90}	10·32	35·62	15·01	79·33	39·40	n/c	55·82	10·27	n/c	12·45
Ave.	4·11	16·09	4·81	29·13	12·61	n/c	14·44	5·26	n/c	3·95
ITR										
IC_{25}	0·02	<0·01	nd†	nd	nd	0·04	nd	<0·01	0·01	nd
IC_{50}	0·06	0·01	<0·01	nd	nd	0·25	<0·01	0·01	0·05	<0·01
IC_{75}	0·20	0·05	0·02	<0·01	<0·01	1·75	0·01	0·03	0·21	0·06
IC_{90}	0·58	0·26	0·13	0·01	<0·01	10·13	0·12	0·12	0·79	0·92
Ave.	0·22	0·08	0·04	<0·01	<0·01	3·04	0·03	0·04	0·26	0·24

*n/c, Not calculated, technical problem.

†nd, Not determined, below lowest concentration tested.

ATCC 22019). Compared to those of natural products, IC levels of antifungal drugs varied among *Candida* strains. For example, ranking of sensitivities of all *Candida* strains to natural compounds was generally similar. However, *Ca. parapsilosis* ATCC 22019 was the least susceptible strain to FLU (average ICs: 29·1 mg l^{-1}), but one of the more sensitive strains to ITR (average ICs: <0·01 mg l^{-1}). Alternatively, *Ca. glabrata* PYCC 2418^T was least susceptible to ITR (average ICs: 3 mg l^{-1}), but most susceptible to AMB (average ICs: 0·7 mg l^{-1}). ITR demonstrated the highest antifungal activity being 10 to >100 times more potent than AMB and FLU, respectively, depending on the types of strains tested. An exception was with the *Ca. glabrata* strain, where AMB was, on average, *c.* 5× more potent than ITR.

The range in IC levels of antifungal drugs for the *C. neoformans* strain fell within that of many of the *Candida* reference strains (Table 3). For example, the IC_{90} levels for AMB, FLU and ITR, 5·11, 12·45 and 0·92 mg l^{-1}, respectively, were similar to those of *Ca. parapsilosis* ATCC 22019, *Ca. albicans* ATCC 90028 and *Ca. lusitaniae* PYCC 2705^T, respectively. However, unlike the *Candida* strains, *C. neoformans* was not more sensitive to the drugs than to the natural products. A few *Candida* strains (i.e. *Ca. albicans* ATCC 10231, two *Ca. parapsilosis* and *Ca. tropicalis*) showed higher IC levels to FLU than *C. neoformans*. Similar to the *Candida*, ITR had highest potency of the drugs to the *C. neoformans*.

Drug/natural product interactions

Group A natural product and drug combinations were mainly additive or synergistic against the *Ca. albicans*, and neutral, additive or synergistic against *C. neoformans* (Table 4). With *Ca. albicans*, benzoic acid, thymol and 2,5-DBA were synergistic when combined with one or more of the antifungal drugs, with thymol synergistic with all three drugs. The most dramatic enhancement of antifungal drug activity was thymol and ITR. This combination inhibited growth of the *Ca. albicans* by 96% with each only at an IC_{25} level in the matrix assay (data not shown). Benzoic acid and 2,5-DBA were synergistic with AMB and ITR. ITR and AMB were most amenable to the enhancement of antifungal activity against the *Ca. albicans* in combination with natural products, showing synergism with three of the natural products. Additionally, with the *Ca. albicans*, FLU was synergistic with one of the natural products, while cinnamic and salicylic acids and 2,3-DBA were either neutral or additive with all drugs.

There were some major differences in antifungal drug and natural product interactions for the *C. neoformans* strain compared with the *Ca. albicans* (Table 4). The

Table 4 Fractional Inhibitory Concentration Indices ($FICI_{90}$) and defined interactions of drug/natural product combinations against *Candida albicans* ATCC 90028 and *Cryptococcus neoformans* CBS 132^T

	Antifungal drug					
	Amphotericin B		Fluconazole		Itraconazole	
Natural prod.	*Ca. albicans*	*C. neoformans*	*Ca. albicans*	*C. neoformans*	*Ca. albicans*	*C. neoformans*
Cinnamic acid	0·54 add*	1·06 neu	0·71 add	2·00 neu	0·64 add	**0·32 syn**
Benzoic acid	**0·27 syn**	0·56 add	0·83 add	1·01 neu	**0·20 syn**	1·00 add
Salicylic acid	0·53 add	1·01 neu	1·40 neu	1·16 neu	0·62 add	1·00 add
Thymol	**0·50 syn**	**0·40 syn**	**0·37 syn**	1·01 neu	**0·21 syn**	1·00 add
2,5-DBA	**0·46 syn**	**0·12 syn**	0·71 add	**0·35 syn**	**0·47 syn**	**0·35 syn**
2,3-DBA	1·61 neu	**0·09 syn**	2·0 neu	1·01 neu	0·79 add	**0·31 syn**

*Interactions: **syn = synergistic**; add = additive; neu = neutral

†DBA, dihydroxybenzaldehyde.

Bold font indicates synergistic (syn) interactions between compounds.

starkest was 2,3-DBA, being synergistic with AMB and ITR against the *C. neoformans*. Alternatively, cinnamic acid, which was additive with all three drugs against the *Ca. albicans*, was neutral with FLU and synergistic with ITR against the *C. neoformans*.

In summary, 2,5-DBA had the broadest synergism with all drugs against both strains, being synergistic with all three drugs against *C. neoformans*, and AMB and ITR against *Ca. albicans*. Thymol also had a synergistic interaction with one or more of the drugs with both strains. Except with salicylic acid, ITR was synergistic with all natural compounds with one or the other strain. AMB also showed predominantly favourable interactions, synergistic with four natural products. FLU, contrastingly, was synergistic with only two compounds.

Discussion

The increase in incidence of candidiases and cryptococcosis combined with resistance and stagnation in development of antifungal agents is a major medical issue. Combination therapy with available drugs has only achieved moderate recognition in view of potential deleterious side effects (Mougdal *et al.* 2005; Clemons *et al.* 2006).

The initial discovery of natural products as chemosensitizing agents involved commercial agricultural fungicides (Kim *et al.* 2006, 2007, 2008b). The mode of action of the fungicides was disruption of redox homeostasis. Accordingly, the most effective chemosensitizing agents were found to target the oxidative stress response system. Certain phenylpropanoids have been reported to have antifungal activity through induction of oxidative stress (Ahmad *et al.* 2010; Khan *et al.* 2010). A synthetic peptide, 4-methoxy-2,3,6-trimethylbenzensulfonyl-substituted D-octapeptide, KN20, that disrupted an ABC-transporter associated with FLU resistance, was reported as a chemosensitizing agent (Niimi *et al.* 2004). However, its fate and toxicity in an *in vivo* system have not since been reported.

2,5-DBA was previously found to synergize a commercial agricultural fungicide (Kim *et al.* 2008b) against *Aspergillus fumigatus*, an agent of invasive aspergillosis (Walsh *et al.* 2008). However, two other benzoic analogues (cinnamic acid, 2,3-DBA) that enhanced agricultural fungicides by targeting the oxidative stress response system in that study had only a neutral interaction with an antifungal drug in this study.

Thymol, a natural product of the common spice, thyme, was used as a traditional, natural antifungal agent in Chinese medicine, but has far lower antifungal activity than commercially available azoles (Nong *et al.* 1999). However, our endeavour is not to use natural products directly as antifungal agents, but use them to enhance activity of available antifungal agents. Synergism by thymol may result from disruption of the cell wall/membrane integrity mitogen-activated protein kinase (MAPK) system (Kim *et al.* 2008a) or by creating lesions in the plasma membrane in combination with disruption of ergosterol biosynthesis by FLU (Guo *et al.* 2009).

Variability in drug/natural product interaction can be affected by type of yeast strain. In our matrix assays, we used strains that had not been exposed to the drugs, and ICs of these strains to the drugs were similarly susceptible. Alternatively, these strains responded to natural products differently. The *Ca. albicans* strain originated from human blood, whereas the *C. neoformans* was collected from fruit juice. Fruit juices contain a number of different phenolic compounds (Hernandez *et al.* 1997), and MAPK stress response pathways among different strains of yeast can respond quite differently to treatment by phenolics (Kim *et al.* 2008a). This may be reflected by the *C. neoformans* strain having seven neutral phenolic/drug

interactions, whereas the *Ca. albicans* had only three, the remaining being additive or synergistic. Some clinical strains of *C. neoformans*, from blood or other human tissues, respond to chemosensitization by these same phenolics (unpublished results).

Chemosensitization may also greatly depend upon drug employed. Different activities between phenolic drug combinations may depend upon the positions simultaneously occupied by hydroxyl groups on the aromatic rings of the phenolics. This would affect dihydroquinone/semiquinone/quinone redox cycling, resulting in different redox activities (Brunmark and Cadenas 1989). This redox activity could affect response of the yeast to the drug or result in a direct chemical interaction between natural compound and drug.

In conclusion, our biologically based study is a proof-of-concept that natural products can enhance antifungal drugs. We have screened the clinical collection of *Candida* and *Cryptococcus* at the *Instituto de Higiene e Medicina Tropical* and have identified a number of drug-resistant strains. Chemosensitization studies of these strains are underway, using clinical laboratory protocols. We have identified combinations of natural product and drug that result in loss of drug resistance in these strains (unpublished results). Indeed, thymol has been shown to be an effective chemosensitizing agent against FLU-resistant clinical strains of *Ca. albicans* (Guo *et al.* 2009). An additional effort has been initiated using animal models. However, our current *in vitro* results point to a promising new approach of improving effectiveness of antifungal drug chemotherapy while lowering dosages, costs and unwanted side effects.

Acknowledgements

This research was conducted under USDA-ARS CRIS Project 5325-42000-035-00D with partial support from the Thomas J. Walsh Award in Clinical Mycology.

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