Synergistic activity of azoles with amiodarone against clinically resistant *Candida albicans* tested by chequerboard and time–kill methods

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Candida albicans is the most common candidal pathogen, causing serious systemic disease in immunocompromised patients. Azoles are widely applied and largely effective; however, they are generally fungistatic and clinically resistant isolates are emerging increasingly. The present study provided *in vitro* evidence using a chequerboard technique that amiodarone is strongly synergistic with azoles against resistant *C. albicans*, with mean fractional inhibitory concentration indices of 0.01 and high-percentage synergistic interactions of 1250 %. A time–kill study performed by both colony counting and a colorimetric reduction assay confirmed the synergistic interaction, with a $\geq 2 \log_{10}$ decrease in c.f.u. ml⁻¹ compared with the corresponding azoles alone. These results suggest the possibility of supplementing azoles with amiodarone to treat resistant *C. albicans* infections.

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INTRODUCTION

Candida albicans is the predominant cause of virtually all types of candidiasis (Filler & Sheppard, 2006). Coinciding with the occurrence of azole-resistant isolates, much effort has been undertaken to solve this by: (i) searching for new antifungal agents, such as posaconazole and ravuconazole, although new drugs for the treatment of these resistant infections are unlikely to appear soon enough and in sufficient numbers (Andes *et al.*, 2006); (ii) developing new formulations of established agents, such as itraconazole (ITC), an intravenous formulation of which has overcome the poor and erratic bioavailability of the oral capsule formulation (Pfaller *et al.*, 2005); and (iii) improving the efficacy of antifungal agents by using combination therapy, for example combining agents with different antifungal mechanisms (Barchiesi *et al.*, 1998; Ghannoum *et al.*, 1995; Girmenia *et al.*, 2003) or combining antifungal and non-antifungal agents (Cruz *et al.*, 2002; Quan *et al.*, 2006).

Recently, the anti-arrhythmic drug amiodarone (AMD) was reported to have potent fungicidal activity against a broad range of fungi (*Cryptococcus*, *Saccharomyces*,

Aspergillus, *Candida* and *Fusarium*) by testing the doubling times or cell weight (Courchesne, 2002). Researchers also showed that low doses of AMD acted synergistically with fluconazole (FLC) against *C. albicans* SC5314 by detecting the post-antifungal effect (Gupta *et al.*, 2003). These results made us interested in a more detailed study of the *in vitro* activity of AMD in combination with azoles [FLC, ITC and voriconazole (VRC)] by conventional methods. In this paper, we present the combined effects of the three azoles and AMD against resistant *C. albicans* by multiple methods of testing and assessing. The strains used were new clinical isolates rather than standard *C. albicans* (Gupta *et al.*, 2003).

METHODS

Strains. New clinical isolates of *C. albicans* strains were used in this study, namely CA10, CA15, CA16, CA135, CA137, CA5, CA8, CA12, CA14 and CA129. All strains were confirmed using standard mycological methods (Baumgartner *et al.*, 1996; Fricker-Hidalgo *et al.*, 1996; Pfaller *et al.*, 1996) in the Microbiological Research Laboratory, Center of Health Research and Epidemic Prevention, Shandong Province, PR China. *Candida parapsilosis* ATCC 22019 was used as a quality-control strain. Isolates were stored at -70 °C, maintained at 4 °C and subcultured twice at 35 °C on Sabouraud dextrose agar.

Drugs. Standard powders of FLC (Shandong Chengchuang Pharmaceutical Technology Development), ITC (Xian-Janssen

Abbreviations: AMD, amiodarone; BI, Bliss independence; CI, confidence interval; FIC, fractional inhibitory concentration; FICI, FIC index; FLC, fluconazole; ITC, itraconazole; LA, Loewe additivity; VRC, voriconazole; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide.

Pharmaceutical), VRC (Chengdu Qihe Pharmaceutical) and AMD hydrochloride (Zhejiang Excel Pharmaceutical) were used in the study. FLC was dissolved in sterile water at a final concentration of 2.56 mg ml⁻¹. ITC, VRC and AMD were each dissolved in DMSO at a final concentration of 25.6 mg ml⁻¹.

Medium. The medium was liquid RPMI 1640 (with L-glutamine, without sodium bicarbonate; Sigma) supplemented with glucose to a final concentration of 2% (Espinel-Ingroff *et al.*, 2005a) and 0.165 M MOPS (Sigma). The pH of the medium was adjusted to 7.0 ± 0.1 at 22 °C with 0.1 M NaOH.

Drug susceptibility testing. MICs were tested according to the method of the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) (CLSI, 2002). The inoculum was adjusted to achieve a final concentration of 0.5 × 10³ to 2.5 × 10³ c.f.u. ml⁻¹. Drug dilutions were prepared in RPMI 1640 and the appropriate range of drug concentrations was chosen by an exploratory study. DMSO was always ≤ 1% of the total volume of medium. Plates were incubated at 35 °C for 48 h. The OD₄₉₂ was measured using a plate reader and background optical densities were subtracted from that of each well. Each isolate was tested in triplicate. MIC was defined as the lowest drug concentration showing 80% (or more) growth reduction compared with the drug-free control. Interpretive breakpoints for FLC [susceptible (S) ≤ 8 µg ml⁻¹; susceptible-dose-dependent (SDD) 16–32 µg ml⁻¹; resistant (R) ≥ 64 µg ml⁻¹], ITC (S ≤ 0.12 µg ml⁻¹; SDD 0.25–0.5 µg ml⁻¹; R ≥ 1 µg ml⁻¹) and VRC (S ≤ 1 µg ml⁻¹; SDD 2 µg ml⁻¹; R ≥ 4 µg ml⁻¹) were used (Espinel-Ingroff *et al.*, 2005b).

Drug interactions. Assays were performed in 96-well flat-bottomed microtitre plates (Costar) using a checkerboard method. The inoculum was used at a final concentration of 0.5 × 10³ to 2.5 × 10³ c.f.u. ml⁻¹ and was verified by colony counting. The final concentrations of the drugs ranged from 0.125 to 128 µg ml⁻¹ for FLC, 0.008 to 8 µg ml⁻¹ for ITC and VRC, and 0.125 to 8 µg ml⁻¹ for AMD. Each isolate was tested three times on different days. In order to assess the nature and intensity of the *in vitro* interactions between the three azoles and AMD, the data obtained were analysed using models based on two non-interaction theories: the Loewe additivity (LA) and the Bliss independence (BI) theories.

The non-parametric approach of LA is based on the fractional inhibitory concentration (FIC) index (FICI) model expressed as: $\Sigma FIC = FIC_A + FIC_B = (\text{MIC of drug A in combination} / \text{MIC of drug A alone}) + (\text{MIC of drug B in combination} / \text{MIC of drug B alone})$ (Te Dorsthorst *et al.*, 2004). Among all of the ΣFIC values calculated for each dataset, the FICI was determined as $\Sigma FIC_{\min}$ (the lowest ΣFIC) when $\Sigma FIC_{\max}$ (the highest ΣFIC) was less than 4; otherwise, the FICI was determined as $\Sigma FIC_{\max}$ (Meletiadis *et al.*, 2003). Synergy and antagonism were defined by FICIs of ≤ 0.5 and > 4, respectively. An FICI result of > 0.5 but ≤ 4 was considered indifferent (Odds, 2003).

The non-parametric approach of BI is based on the Prichard model defined as $E_i = E_A \times E_B$, where E_i is the predicted percentage of growth of the theoretical combination of drugs A and B, respectively, and E_A and E_B are the experimental percentages of growth of each drug acting alone, respectively. Interaction is described by the difference (ΔE) between the predicted and measured percentages of growth at various concentrations. Because of the nature of the interaction testing with microtitre plates and a twofold dilution of either drug, this results in a ΔE for each drug combination. When the mean ΔE was positive and its 95% confidence interval (CI) among the replicates did not include 0, statistically significant (SS) synergy was claimed; when the ΔE was negative and its 95% CI did not include 0, SS antagonism was claimed. In any other case, BI was concluded. The ΔE values obtained for each combination can be depicted on the z-axis to construct a 3D surface plot. Peaks above and below the 0 plane indicate synergistic

and antagonistic combinations, respectively, whilst the 0 plane indicates the absence of SS interaction. As the plot only shows the interactions for each separate combination of the concentrations, a value is needed to summarize the interaction surface. This was achieved by calculating the sum percentage of all SS synergistic (ΣSYN) and antagonistic (ΣANT) interactions. Interactions with < 100% of SS interactions were considered weak, those with 100–200% of SS interactions were considered moderate, and those with > 200% of SS interactions were considered strong. In addition, the numbers of SS synergistic and antagonistic combinations among the 77 combinations tested were calculated for each strain (Afeltra *et al.*, 2004).

Time-kill assay. We investigated the activity of AMD with or without azoles against one resistant strain (CA10) at a starting inoculum of 10⁵ c.f.u. ml⁻¹. A set of solutions was prepared: a growth control (drug-free), AMD alone (4 µg ml⁻¹), azoles alone (8 µg ml⁻¹ for FLC, 0.5 µg ml⁻¹ for ITC and VRC) and combinations of AMD with each azole. All solutions were incubated at 35 °C without agitation (Klepser *et al.*, 1998). At predetermined time points (0, 6, 12, 24 and 48 h), a 100 µl sample was removed from each solution and serially diluted. A 100 µl aliquot from each dilution was then streaked onto a Sabouraud dextrose agar plate to perform colony counting after incubation for 48 h. A colorimetric reduction assay was carried out using 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT; Sigma). XTT was dissolved at 0.5 g l⁻¹ in Ringer's lactate, sterilized through a 0.22 µm filter, stored at -70 °C and maintained at 4 °C when in use. Before use, 100 mM menadione in acetone was added to a final concentration of 10 µM (Ramage *et al.*, 2001). At the same predetermined time points, another 100 µl sample was removed from each solution into a 96-well flat-bottomed microtitre plate and a 100 µl aliquot of XTT/menadione solution was added to each well. The plate was incubated in the dark for up to 2 h at 35 °C. After this, the absorbance of the XTT reduction product, formazan, was read at 492 nm for each well using a plate reader (Lewis & Kontoyiannis, 2005). All experiments were conducted in triplicate.

A standard correlation between viable cell counts from colony counting and optical density values from the XTT reduction assay was used to transform XTT reduction results into cell counts and to compare them. The following criteria were used for both methods to interpret time-kill results: synergy, a ≥ 2 log₁₀ decrease in c.f.u. ml⁻¹ compared with the most active constituent; antagonism, a ≥ 2 log₁₀ increase in c.f.u. ml⁻¹ compared with the least active agent; additivity, a < 2 but > 1 log₁₀ decrease in c.f.u. ml⁻¹ compared with the most active agent; and indifference, a < 2 but > 1 log₁₀ increase in c.f.u. ml⁻¹ compared with the least active agent (Mukherjee *et al.*, 2005).

RESULTS AND DISCUSSION

Susceptibility and interaction of drugs

The MICs of FLC, ITC and VRC in each test, based on an 80% reduction in growth, for *C. parapsilosis* ATCC 22019 were 4, 0.125 and 0.063 µg ml⁻¹, respectively, which are within the reference ranges. Table 1 summarizes the *in vitro* susceptibility of the drugs alone and in combination against *C. albicans*. The susceptibility of experimental isolates agreed well among the three azoles, indicating the existence of cross-resistance. Generally, for cross-resistant strains, the FICI ranged from 0.001 to 0.018 (much smaller than 0.5) and the percentages of synergistic interactions ranged from 691 to 2256% (far beyond 100%), suggesting

Table 1. Susceptibility of drugs alone and in combination against clinically resistant *C. albicans*

The MIC and FICI values are shown as the median of three independent experiments. R, Resistant; S, susceptible; INT, interpretation; SYN, synergism; ANT, antagonism; NI, no interaction; *n*, number of interactions.

Drug and strain	MIC ($\mu\text{g ml}^{-1}$)			LA theory		BI theory		
	Azole	AMD	INT	FICI	INT	ΣSYN (<i>n</i>)	ΣANT (<i>n</i>)	INT
FLC								
CA10	256	512	R	0.008	SYN	1494 (54)	−18 (4)	SYN
CA15	128	512	R	0.014	SYN	1216 (56)	−23 (6)	SYN
CA16	128	512	R	0.014	SYN	1322 (65)	0 (0)	SYN
CA135	64	512	R	0.008	SYN	805 (61)	−6 (3)	SYN
CA137	64	512	R	0.008	SYN	1027 (63)	−72 (7)	SYN
CA5	0.25	512	S	4.02	ANT	43 (14)	−430 (16)	ANT
CA8	0.25	512	S	1.00	NI	90 (26)	−196 (30)	ANT
CA12	4	512	S	1.00	NI	79 (27)	−163 (25)	ANT
CA14	0.5	512	S	1.00	NI	17 (13)	−8 (3)	NI
CA129	0.5	512	S	1.00	NI	37 (16)	−18 (4)	NI
ITC								
CA10	128	512	R	0.011	SYN	1302 (61)	−33 (9)	SYN
CA15	16	512	R	0.018	SYN	1220 (56)	−18 (6)	SYN
CA16	32	512	R	0.014	SYN	1327 (55)	−89 (12)	SYN
CA135	16	512	R	0.008	SYN	1312 (59)	0 (0)	SYN
CA137	16	512	R	0.008	SYN	738 (47)	−10 (5)	SYN
CA5	0.031	512	S	4.02	ANT	12 (4)	−301 (17)	ANT
CA8	0.016	512	S	4.02	ANT	98 (34)	−54 (10)	NI
CA12	0.063	512	S	4.02	ANT	79 (17)	−197 (25)	ANT
CA14	0.063	512	S	0.50	SYN	153 (31)	−422 (21)	ANT
CA129	0.063	512	S	1.00	NI	108 (18)	−74 (11)	SYN
VRC								
CA10	64	512	R	0.001	SYN	2256 (66)	−8 (5)	SYN
CA15	8	512	R	0.012	SYN	1562 (53)	0 (0)	SYN
CA16	8	512	R	0.014	SYN	1704 (59)	−3 (1)	SYN
CA135	8	512	R	0.002	SYN	691 (54)	0 (0)	SYN
CA137	8	512	R	0.008	SYN	779 (66)	0 (0)	SYN
CA5	0.016	512	S	1.00	NI	0 (0)	−5 (5)	NI
CA8	0.008	512	S	1.00	NI	91 (25)	−88 (19)	NI
CA12	0.031	512	S	2.00	NI	63 (13)	−87 (7)	NI
CA14	0.008	512	S	1.00	NI	15 (7)	−16 (2)	NI
CA129	0.016	512	S	1.00	NI	0 (0)	−21 (1)	NI

a very strong enhanced antifungal effect of azoles by AMD, whilst there was predominantly no synergistic effect observed for the tested susceptible *C. albicans* isolates. To obtain a visual characteristic of the drug combinations, a 3D surface plot of FLC combined with AMD against strain CA10 was prepared (Fig. 1).

In vitro interaction of two or more agents is dependent on the methodology used to generate the data, as well as on the way the results are analysed, resulting in variable as well as controversial conclusions (Meletiadiis *et al.*, 2003). Compared with the FICI, the Prichard model not only allows an objective statistical criterion, but also fits all the experimental concentrations to construct a 3D graph to visualize the nature and intensity of drug combinations without arbitrarily choosing an end point (Te Dorsthorst *et al.*, 2002). Compared with the fully parametric and

semi-parametric response surface approaches (Afeltra *et al.*, 2004), the Prichard model is not dependent on the data analysis program and the sigmoid dose–response, and thus does not fail to fit the data. Last but most importantly, the Prichard model had an excellent reproducibility for the 77 combinations calculated for each strain. Current methods based on the LA and BI models are associated with implicit assumptions about the interacting system (Tam *et al.*, 2004). Considering all of the above, we strongly advise the use of the Prichard model to assess the interaction between two drugs for its peerless advantages.

Time–kill curves of drug combinations

The optical density values from the XTT reduction assay were transformed into colony counts using the standard

Fig. 1. Three-dimensional plot of the synergy of FLC in combination with AMD against strain CA10 using the MATLAB program. The mean ΔE values obtained for three separate experiments were depicted on the z-axis to form the graph. Peaks above the 0 plane indicate synergistic combination.

curve in Fig. 2. The results of AMD combined with the azoles from the time-kill study are shown in Table 2. Synergistic interactions from chequerboard testing were confirmed by time-kill curves, although we had to convert the colony counting data from FLC with AMD and ITC with AMD into integers. Representative plots of $\log_{10}$ c.f.u. ml^{-1} against time for the XTT reduction assay are shown in Fig. 3. AMD alone had a very weak antifungal effect at $4 \mu\text{g ml}^{-1}$ at 48 h, but the fungistatic activity of azoles against the resistant strain (CA10) was dramatically enhanced by the addition of AMD. Given the initial inoculum of 10^5 c.f.u. ml^{-1} , the combination yielded, respectively, a mean decrease of 2.02, 2.04 and 2.08 $\log_{10}$ c.f.u. ml^{-1} for FLC, ITC and VRC after incubation for 48 h. There were no obvious differences in features of drug action among the three combinations.

Time-kill curves provided growth kinetic information over time and gave a more detailed picture of the effect of drug combinations on cell viability (Mukherjee *et al.*, 2005). XTT is reduced by mitochondrial dehydrogenases of viable

fungi into a water-soluble formazan product, which is measured by the absorbance at 492 nm (Kuhn *et al.*, 2002). Previous work showed an actual relationship between *Candida* cell number and XTT formazan signal (Kuhn *et al.*, 2003), so we carried out time-kill experiments using the XTT reduction assay and compared it with colony counting. Without the influence of filament formation, time-kill curves obtained using the XTT assay were as reliable as colony counting. The XTT assay remains a valuable tool for examining the growth and metabolism of yeast, whether in planktonic or biofilm form (Kuhn *et al.*, 2003), and may become a substitute for colony counting for its time-saving advantage.

Potential mechanisms of AMD that augment the antifungal effects of azoles

The action of azoles not only involves impeding ergosterol biosynthesis through the competitive inhibition of 14α -demethylase, which is a product of the *ERG11* gene, but

Fig. 2. Relationship between colony counts and results of the XTT reduction assay to measure the growth of *C. albicans* without drugs after 48 h. Results are expressed as $y=4.261x+4.2087$, with correlation coefficient $r=0.9622$.

Table 2. Decrease in c.f.u. ml^{-1} of strain CA10 using AMD combined with azoles at 48 h

The combined decrease in c.f.u. ml^{-1} was compared with that of the corresponding azoles, and the triplicate results of experiments are reported as mean $\pm$ SD. INT, Interpretation; SYN, synergism.

Drug combination	Colony count		XTT reduction assay	
	Mean $\pm$ SD	INT	Mean $\pm$ SD	INT
FLC+AMD	1.99 ± 0.02	SYN	2.02 ± 0.03	SYN
ITR+AMD	1.99 ± 0.02	SYN	2.04 ± 0.03	SYN
VRC+AMD	2.02 ± 0.02	SYN	2.08 ± 0.03	SYN

Fig. 3. Time-kill curves for strain CA10 obtained by XTT assay using $8 \mu\text{g ml}^{-1}$ for FLC (a) and $0.5 \mu\text{g ml}^{-1}$ for ITC (b) and VRC (c) combined with AMD at $4 \mu\text{g ml}^{-1}$. ■, Growth control; □, AMD alone; ▲, azole alone; △, azole combined with AMD.

also leads to accumulation of 14-methylated sterol, which can cause disruption of fungal membranes (Morschhäuser, 2002; Ribeiro & Paula, 2007). Our investigation illustrated that AMD used in combination with azoles acted synergistically against resistant *C. albicans* strains, whilst there was almost no synergistic activity seen against susceptible strains. Considering the effect of AMD in inhibiting L-type Ca^{2+} channels and Na^+/K^+ channels when treating atrial fibrillation in patients with left ventricular dysfunction and heart failure, we propose that the most likely mechanism of the synergistic effect would be a consequence of disrupted Ca^{2+} homeostasis as reported by Gupta *et al.* (2003). Other possible mechanisms whereby AMD could reduce the overexpression of genes encoding efflux pumps or azole target enzymes are still under study.

Although AMD can cause serious adverse effects, including anaemia (Nicolay *et al.*, 2007), corneal microdeposits, optic neuropathy/neuritis, blue-grey skin discoloration, photosensitivity, hypothyroidism, hyperthyroidism, pulmonary toxicity, peripheral neuropathy and hepatotoxicity (Vassallo & Trohman, 2007), as one of the first-line agents

of atrial fibrillation treatment, it is widely used in clinical situations. In addition, this study provides a new clue to antifungal mechanisms by considering the known actions of AMD, and suggests a way of treating resistant *C. albicans* infection through a drug combination approach.

In conclusion, our results highlight the promising role of AMD used in combination with azoles to treat serious resistant *C. albicans* infection. Prospective studies in appropriate animal models are required to develop therapeutic strategies for these combinations, and further studies in other types of fungi and with a larger number of strains are needed.

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