Infection and Drug Resistance

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ORIGINAL RESEARCH

Cotreatment with Aspirin and Azole Drugs Increases Sensitivity of *Candida albicans* in vitro

Wenli Feng
Jing Yang
Yan Ma
Zhiqin Xi
Ying Ji
Qiao Ren
Huan Ning
Shaoyan Wang

The Department of Dermatovenereology, The Second Hospital of Shanxi Medical University, Taiyuan, 030001, Shanxi, People's Republic of China

Purpose: This study aimed to investigate the effects of aspirin (acetyl salicylic acid [ASA]) combined with fluconazole (FCA), itraconazole (ITR), or voriconazole (VRC) on *Candida albicans* under planktonic and biofilm conditions.

Methods: A total of 39 clinical *C. albicans* strains were used to perform the in vitro drug sensitivity assay under different conditions using the M27-A4 broth microdilution method. The minimal inhibitory concentrations (MICs) and fractional inhibitory concentration index (FICI) values were calculated. *C. albicans* ZY23 was chosen for the further analyses.

Results: Under planktonic conditions, the half maximal MIC (MIC_{50}) values of FCA, ITR, and VRC were 64–0.5 μg/mL, 32–0.0625 μg/mL, and 16–0.125 μg/mL, respectively, when applied, whereas in combination with ASA, the values decreased to 32–0.25 μg/mL, 8–0.0313 μg/mL, and 8–0.0313 μg/mL, respectively. Under biofilm conditions, FCA, ITR, or VRC alone showed MIC_{50} values of 128–8 μg/mL, 32–4 μg/mL, and 32–0.5 μg/mL, whereas in combination with ASA the values were decreased to 32–0.5 μg/mL, 16–0.5 μg/mL, and 8–0.0625 μg/mL, respectively. Analysis of the FICI showed that the sensitization rate of ASA to FCA, ITR, and FCA under planktonic conditions was 43.59%, whereas the sensitization rates of ASP to FCA, ITR, and FCA under biofilm conditions were 46.15%, 46.15%, and 48.72%, respectively. Additionally, the time-growth and time-kill curves of *C. albicans* ZY23 further verified the synergistic effects of ASA on azole drugs.

Conclusion: ASA may act as an enhancer of the inhibitory effects of azole drugs on the growth of clinical *C. albicans* under planktonic and biofilm conditions.

Keywords: acetylsalicylic acid, synergistic effects, invasive fungus, virulence

Introduction

Invasive fungi have become an important cause of serious and fatal infections over the past few decades, and the associated infections cause dangerous diseases with rapid clinical progression, poor prognosis, and high fatality rates.[1] Candidiasis, mainly caused by *Candida albicans*, is a serious invasive fungal infection that is ranked as the fourth most prevalent nosocomial bloodstream infections in hospitals, with a mortality rate of up to 50%.[2–5] The severity of *C. albicans* infections is closely related to virulence factors, such as phenotypic transformation, invasive enzymes, adhesion factors, and host and environmental factors.[6] Currently, only a few types of antifungal agents including azoles, echinocandins, polyenes, and allylamines are available for the management of *C. albicans* infections.[7] Fluconazole (FCA), itraconazole (ITR), and voriconazole (VRC) are azole drugs commonly used for the treatment of infections caused by *C. albicans* because of their high bioavailability.[8] However, the

Correspondence: Wenli Feng; Jing Yang
The Department of Dermatovenereology, The Second Hospital of Shanxi Medical University, No. 382, Wuyi Road, Taiyuan, Shanxi, 030001, People's Republic of China
Tel +86-0351-3365410
Email wenlifeng2010@163.com; yangjing7962@126.com

extensive and frequent use of azole drugs has gradually increased the number of drug-resistant strains of *C. albicans*, which has become a great challenge in the treatment of fungal infections. In addition, *C. albicans* easily form biofilms on medical devices, which can reduce its susceptibility to drugs and is thought to be physiologically related to the acquisition of drug resistance against antifungal agents.[9,10] Therefore, drug resistance has become an important challenge in the treatment of *C. albicans* infections. There is an urgent need to develop improved and novel antifungal therapies to reduce drug resistance of *C. albicans* in treating candidiasis.

Candidiasis can cause host cells to release proinflammatory cytokines and large amounts of arachidonic acid (AA). Subsequently, AA can be converted to eicosanoids by lipoxygenases and cyclooxygenases (COXs), and prostaglandins (PGs) may play an important role in fungal colonization.[11] Non-steroidal anti-inflammatory drugs (NSAIDs) are inhibitors of COX-1 and COX-2 isoenzymes. The NSAID aspirin (acetylsalicylic acid [ASA]) blocks the production of PG E2 (PGE2) by inhibiting the activity of COXs, and PGE2 promotes the formation of fungal hyphae.[12] Additionally, ASA has been reported to increase the sensitivity of FCA-resistant strains of *C. albicans*, reduce the adhesion of *C. albicans* to abiotic surfaces, and inhibit the growth of *C. albicans* in the planktonic state.[13] Rusu et al[14] reported that ASA effectively reduced the formation of germ tubes by *C. albicans* and inhibited fungal viability. Previous studies have shown that in combination with amphotericin B, ASA influences the formation of biofilms of *C. albicans* by upregulating its glucoamylase 1 (GAM1) homolog *GCA1* and downregulating cell division cycle mutant 35 (*CDC35*), *CSR1*, enhanced filamentous growth protein 1 (*EFG1*), and hyphal wall protein 1 (*HWP1*), thus, improving the efficacy of amphotericin B.[15,16] However, the combined effects of ASA and azole drugs on *C. albicans* remain unclear.

In this study, biofilms of a clinical *C. albicans* strain were formed, and in vitro drug sensitivity tests were conducted using the M27-A4 broth microdilution method under planktonic and biofilm conditions. Based on the results of drug sensitivity, the enhancement of the growth inhibiting effects of azole drugs by ASA was explored. These findings provide potential novel approaches for the clinical treatment of diseases caused by *C. albicans* infection.

Materials and Methods

Experimental Strains

A total of 39 clinical strains of *C. albicans* were provided by the Fungal Laboratory, Department of Dermatology and The Second Hospital of Shanxi Medical University (Shanxi, China), and their origins are shown in Table 1. In addition, a standard strain of *C. albicans* (ATCC 11006) and quality control strains (*Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019) were purchased from the Fungus and Mycosis Research Center, Department of Medicine, Peking University (Beijing, China).

Preparation of Drug Stock Solutions

ASA, FCA, ITR, and VRC were purchased from Beijing Runzekang Biological Technology Co., Ltd. (Beijing, China). The stock solution of ASA (32 mg/mL) was prepared by dissolving 160 mg ASA powder in 5 mL dimethyl sulfoxide (DMSO, Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China). Furthermore, 6.4 mg of FCA powder was dissolved in 5 mL sterilized double distilled water, and a stock solution of a concentration of 1280 μg/mL was prepared. Additionally, 3.2 mg ITR or VRC was dissolved in 1 mL DMSO, and a stock solution of each agent at a concentration of 3200 μg/mL was prepared. All stock solutions were stored at −20°C.

Preparation of Fungal Suspension Under Planktonic and Biofilm Conditions

C. albicans was inoculated into yeast extract peptone dextrose (YPD) liquid medium (Saipuruisi Beijing Technology Co., Ltd., Beijing, China), cultured overnight, and then 1 mL of the fungal suspension was centrifuged at high speed for 2 min and then washed twice with saline. Then, *C. albicans* was resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Saipuruisi Beijing Technology Co., Ltd.) and diluted to 1×10^3 CFU/mL. A fungal suspension was prepared to measure drug sensitivity under planktonic conditions.

Fungal suspensions were prepared under biofilm conditions as previously described.[17,18] The experimental strains were inoculated into a YPD agar medium (Saipuruisi Beijing Technology Co., Ltd.), cultured at 37°C for 72 h, and then a fresh single colony was selected for inoculation into YPD liquid medium, followed by culturing at 30°C for 24 h with agitation at 200 rpm. The fungal suspension (1 mL) was collected into a new 1.5 mL tube

Table 1 The Clinical Features of the Patients Where the Strains Were Isolated

Strain (ZY)	Gender	Age	Source	Symptom
1	Female	53	Urine	Dysuria, urgency, and frequency
4	Female	61	Sputum	Fever, cough and sputum
7	Male	55	Faeces	Diarrhea
9	Male	56	Sputum	Fever, cough and sputum
11	Female	65	Sputum	Fever, cough and sputum
12	Female	65	Sputum	Fever, cough and sputum
15	Female	76	Sputum	Fever, cough and sputum
18	Male	24	Sputum	Fever, cough and sputum
20	Female	86	Sputum	Fever, cough and sputum
21	Female	56	Sputum	Fever, cough and sputum
22	Female	56	Sputum	Fever, cough and sputum
23	Female	40	Urine	Urgency, and frequency
24	Male	78	Urine	Dysuria, urgency, and frequency
26	Female	62	Urine	Dysuria, urgency, and frequency
27	Female	53	Sputum	Fever, cough and sputum
28	Female	26	Sputum	Fever, cough and sputum
30	Male	72	Sputum	Fever, cough and sputum
33	Female	30	Sputum	Fever, cough and sputum
35	Male	48	Sputum	Fever, cough and sputum
36	Male	88	Sputum	Fever, cough and sputum
38	Female	45	Sputum	Fever, cough and sputum
39	Male	19	Sputum	Fever, cough and sputum
40	Female	71	Faeces	Diarrhea
41	Female	45	Vaginal secretion	Increased vaginal discharge accompanied by pruritus
42	Female	19	Vaginal secretion	Increased vaginal discharge accompanied by pruritus
43	Female	38	Vaginal secretion	Increased vaginal discharge accompanied by pruritus
44	Female	50	Vaginal secretion	Increased vaginal discharge accompanied by pruritus
45	Female	22	Vaginal secretion	Increased vaginal discharge accompanied by pruritus
46	Female	55	Vaginal secretion	Increased vaginal discharge accompanied by pruritus
47	Female	30	Vaginal secretion	Increased vaginal discharge accompanied by pruritus
48	Female	29	Vaginal secretion	Increased vaginal discharge accompanied by pruritus
50	Female	48	Vaginal secretion	Increased vaginal discharge accompanied by pruritus
51	Female	60	Vaginal secretion	Increased vaginal discharge accompanied by pruritus

(*Continued*)

Table 1 (Continued).

Strain (ZY)	Gender	Age	Source	Symptom
52	Female	27	Vaginal secretion	Increased vaginal discharge accompanied by pruritus
53	Female	55	Vaginal secretion	Increased vaginal discharge accompanied by pruritus
55	Male	67	Urine	Dysuria, urgency, and frequency
56	Male	24	Urine	Dysuria, urgency, and frequency
63	Female	72	Urine	Dysuria, urgency, and frequency
64	Male	58	Urine	Dysuria, urgency, and frequency

and centrifuged at 5000 rpm for 2 min. The sediment was washed with saline three times, resuspended in RPMI 1640 medium, and then 100 μL of the fungal suspension was added to each well of a 96-well plate, whereas100 μL RPMI1640 medium was used as a negative control. After culturing at 37°C for 1 h, sterile phosphate-buffered saline (PBS) was added to remove the free cells, and then 100 μL of fresh RPMI1640 medium was added. After culturing at 37°C for 24 h, the fungi were washed with PBS three times, the medium was removed, and then the fungi were incubated at 37°C for another 24 h to form mature biofilms.

Drug Sensitivity Assays Under Different Conditions

A two-fold dilution method was used to prepare the ASA, FCA, ITR, and VRC solutions in RPMI medium. For the experiments under planktonic conditions, the concentrations of ASA, FCA, ITR, and VRC used were from 16 mg/mL to 0.25 mg/mL, 64 μg/mL to 0.125 μg/mL, 16 μg/mL to 0.0313 μg/mL, and 16 μg/mL to 0.0313 μg/mL, respectively. In addition, under biofilm conditions, the concentrations of ASA, FCA, ITR, and VRC used ranged from 32 mg/mL to 0.5 mg/mL, 128 μg/mL to 0.25 μg/mL, 32 μg/mL to 0.0625 μg/mL, and 32 μg/mL to 0.0625 μg/mL, respectively.

Drug sensitivity was analyzed in vitro using the Clinical Laboratory and Standards Institute (CLSI) standard M27-A4 broth microdilution method.[19]

Under planktonic conditions, each concentration of FCA, ITR, or VRC (100 μL) was added to the specified wells of a 96-well plate, followed by different concentrations of ASA (100 μL) and then 100 μL of the fungal suspension was added to each well (except for the negative control). RPMI medium was used as the negative control, and the fungal suspension without any treatment was used as the positive control. Under biofilm conditions, the *C. albicans* biofilm was successfully established on a 96-well plate, and then the drug treatment was similar to that under planktonic conditions. After culturing at 37°C for 48 h, the minimal inhibitory concentrations (MICs) were calculated based on the growth of *C. albicans* on a 96-well plate as previously described.[20,21]

Interpretation of Fractional Inhibitory Concentration Index (FICI)

The fractional inhibitory concentration index (FICI) was used to determine the interaction of the two drugs in combination, and the results were interpreted according to the methods of Tamura et al[22] and Odds et al.[23] The following formula was used for the calculation: FICI= (MIC_A in combination/MIC_A alone) + (MIC_B in combination/MIC_B alone). The effects of the antifungal drug combinations were classified according to the following criteria: (1) FICI ≤ 0.5, synergistic effects; (2) 0.5 < FICI ≤ 1, additive effects; (3) 1 < FICI < 4, no interactions; (4) FICI ≥ 4.0, antagonistic effects.

Time-Growth and Time-Kill Curve Assays

C. albicans ZY23 was chosen to perform the time-growth and time-kill assays to construct the respective curves. The overnight cultured ZY23 suspension was washed with PBS three times and resuspended in a YPD liquid medium to a final concentration of 1×10^4 CFU/mL. The concentrations of ASA, FCA, ITR, and VRC were selected based on the results of the drug sensitivity experiment. The control group consisted of

0.5 mL fungal suspension and 4.5 mL RPMI medium. The single-drug group was 0.5 mL fungi suspension, 0.5 mL FCA (8 μg/mL)/ITR (4 μg/mL)/VRC (4 μg/mL), and 4 mL RPMI 1640 medium. The combination groups consisted of 0.5 mL fungi suspension, 0.5 mL FCA (8 μg/mL)/ITR (4 μg/mL)/VRC (4 μg/mL), 0.5 mL different concentrations of ASA (1, 2, 4, and 8 mg/mL), and 3.5 mL RPMI 1640 medium. After incubation at 37°C with oscillation at 200 rpm for 0, 12, 36, and 48 h, the absorbance at 630 nm was measured using a microplate reader, and the curve was plotted to record the growth of *C. albicans* ZY22 at each time point.[24]

For the time-kill curve experiments,[25] *C. albicans* ZY23 was cultured in YPD liquid medium for 16 h, and then the fungal suspension (100 μL) was transferred to fresh YPD liquid medium (10 mL). After culturing for another 4 h, the fungi were washed with PBS three times and resuspended in RPMI medium to a final concentration of 1×10^3 CFU/mL. The control group consisted of a 0.5 mL fungal suspension, a 0.5 mL DMSO, and a 4 mL RPMI medium. The single-drug groups contained 0.5 mL fungal suspension with 0.5 mL FCA (8 μg/mL), ITR (4 μg/mL), or VRC (4 μg/mL) and 4 mL RPMI medium. The combination groups included 0.5 mL fungi suspension; 0.5 mL FCA, ITR, or VRC; 0.5 mL ASA; and 3.5 mL RPMI medium. The mixture was incubated at 30°C with oscillation at 220 rpm. After culturing for 0, 12, 24, 36, and 48 h, the fungal suspension was diluted using a 10-fold dilution method, and then coated on Sabouraud dextrose agar (SDA) medium (Saipuruisi Beijing Technology Co., Ltd.). After culturing at 30°C for 48 h, the fungal colonies were counted and the $\log_{10}$ CFU/mL values were used to draw the curves for the analysis.

Statistical Analysis

Data are presented as means ± standard deviation (SD) and the statistical package for the social sciences (SPSS) software (version 17.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. Differences between the two groups were analyzed using the Student's *t*-test and a one-way analysis of variance (ANOVA) was used to compare more than two groups, while differences were considered statistically significant at $p < 0.05$.

Results

Drug Sensitivity Under Free and Biofilm Conditions

The CLSI standard M27-A4 broth microdilution method was used to determine the MIC_{50} of ASA, FCA, ITR, and VRC against classical *C. albicans* strains under planktonic and biofilm conditions. The MIC_{50} values under different conditions are shown in Tables 2, 3 and S1–S4. The results showed that the MIC_{50} of FCA alone was 64–0.5 μg/mL and 128–8 μg/mL under planktonic and biofilm conditions, respectively, whereas in combination with ASA the values were decreased to 32–0.25 μg/mL, and 32–0.5 μg/mL, respectively (Tables 2 and 3).

Under planktonic conditions, the MIC_{50} values of ITR and VRC alone were 32–0.0625 μg/mL and 16–0.125 μg/mL, respectively, whereas the values decreased to 8–0.0313 μg/mL and 8–0.0313 μg/mL, respectively, in combination with ASA (Tables S1 and S3). In addition, under biofilm conditions, the MIC_{50} values of ITR and VRC alone were 32–4 μg/mL and 32–0.5 μg/mL, respectively, whereas cotreatment with ASA decreased the values to 16–0.5 μg/mL and 8–0.0625 μg/mL, respectively (Tables S2 and S3). These results indicate that compared to treatment with FCA, ITR, or VRC alone, co-treatment with ASA decreased the MIC_{50} values of the agents against clinical *C. albicans* under planktonic and biofilm conditions.

Interaction of FCA, ITR, or VRC with ASA Against *C. albicans*

FICI values were calculated to further analyze the effects of the interaction between ASA and FCA, ITR, or VRC on the sensitivity of *C. albicans*. Synergistic interactions between FCA and ASA under planktonic and biofilm conditions were observed in 17 and 18 strains, respectively, with $FICI \leq 0.5$, additive interactions were observed in 17 and 17 strains, respectively, with $0.5 < FICI \leq 1$; and 5 and 4 strains, respectively, showed no interaction (Table 4). For ITR and ASA, synergistic interactions were found in 17 and 18 strains under planktonic and biofilm conditions, respectively, whereas additive interactions were observed in 18 and 17 strains, respectively (Table 4).

Additionally, under planktonic conditions, synergistic and additive interactions were observed between ASA and VRC in 17 strains each and no interaction was observed in 5 strains. In contrast, under biofilm conditions, synergistic and additive interactions were observed in 19 and 16 strains, respectively, whereas no interaction was observed in 4 strains (Table 4). In summary, the rate at which ASA enhanced the potency of FCA, ITR, and FCA under planktonic conditions was 43.59%; whereas corresponding

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Table 2 The Values of Minimum Inhibitory Concentration (MIC_{50}) Under Planktonic Conditions When Aspirin (ASA) and Fluconazole (FCA) Were Used

		MIC_{50} (μg/mL)						MIC_{50} (μg/mL)			
		ASA		FCA				ASA		FCA	
Strain (ZY)	FICI	Alone	Combination	Alone	Combination	Strain (ZY)	FICI	Alone	Combination	Alone	Combination
1	0.750	16,000	4000	64	32	38	0.500	8000	250	0.5	0.25
4	0.500	16,000	4000	16	8	39	0.125	16,000	500	64	8
7	0.125	16,000	4000	8	1	40	0.250	4000	2000	4	1
9	0.250	8000	2000	64	16	41	0.501	16,000	4000	64	32
11	0.500	8000	2000	4	2	42	0.126	8000	4000	16	2
12	0.504	4000	2000	64	0.25	43	0.127	16,000	8000	16	2
15	1.000	16,000	8000	32	16	44	0.250	16,000	2000	16	4
18	0.504	8000	4000	64	0.25	45	0.250	4000	2000	64	16
20	0.063	4000	500	64	4	46	0.125	8000	1000	32	4
21	0.126	4000	2000	16	2	47	0.501	8000	4000	64	32
22	1.125	8000	8000	64	8	48	0.251	8000	4000	64	16
23	1.000	16,000	8000	16	8	50	0.125	8000	1000	16	2
24	0.125	4000	1000	16	2	51	0.250	4000	1000	4	1
26	0.031	8000	500	16	0.5	52	0.126	16,000	4000	64	8
27	0.625	16,000	2000	4	2	53	0.032	4000	4000	8	0.25
28	0.313	4000	250	32	8	55	0.500	16,000	1000	32	16
30	1.000	8000	4000	8	4	56	0.064	16,000	8000	64	4
33	0.625	16,000	8000	64	8	63	0.125	4000	500	16	2
35	0.500	2000	500	32	8	64	0.500	8000	500	32	16
36	0.500	1000	250	2	0.5						

Abbreviation: FICI, fractional inhibitory concentration index.

Table 3 The Values of Minimum Inhibitory Concentration (MIC_{50}) Under Biofilm Conditions When Aspirin (ASA) and Fluconazole (FCA) Were Used

		MIC_{50} (μg/mL)						MIC_{50} (μg/mL)			
		ASA		FCA				ASA		FCA	
Strain (ZY)	FICI	Alone	Combination	Alone	Combination	Strain (ZY)	FICI	Alone	Combination	Alone	Combination
1	0.500	32,000	8000	64	16	38	0.500	16,000	4000	8	2
4	0.375	16,000	4000	16	2	39	0.750	32,000	16,000	64	16
7	0.375	32,000	8000	16	2	40	0.188	16,000	2000	8	0.5
9	0.625	16,000	2000	64	32	41	1.000	16,000	8000	64	32
11	0.750	16,000	4000	8	4	42	0.500	8000	2000	16	4
12	1.000	8000	4000	64	32	43	1.004	16,000	16,000	128	0.5
15	0.500	16,000	4000	32	8	44	0.625	16,000	8000	32	4
18	0.625	8000	4000	128	16	45	0.625	8000	1000	64	32
20	0.375	16,000	2000	64	16	46	0.750	16,000	8000	32	8
21	0.250	8000	1000	32	4	47	0.625	16,000	2000	64	32
22	0.625	16,000	2000	32	16	48	1.000	8000	4000	32	16
23	1.000	16,000	8000	64	32	50	0.375	16,000	4000	32	4
24	1.250	16,000	16,000	32	8	51	0.375	8000	1000	8	2
26	0.188	32,000	2000	32	4	52	1.250	16,000	16,000	64	16
27	0.750	16,000	8000	8	2	53	0.563	8000	4000	16	1
28	0.750	8000	4000	64	16	55	0.375	16,000	4000	32	4
30	1.000	16,000	8000	8	4	56	0.750	16,000	8000	128	32
33	1.500	16,000	16,000	64	32	63	0.313	8000	2000	16	1
35	1.000	4000	2000	32	16	64	0.625	16,000	2000	16	8
36	0.625	8000	1000	8	4						

Abbreviation: FICI, fractional inhibitory concentration index.

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Table 4 The Interactions Between Aspirin (ASA) and Fluconazole (FCA), Itraconazole (ITR) or Voriconazole (VRC) Under Different Conditions

	ASA Combined with FCA		ASA Combined with ITR		ASA Combined with VRC	
	Planktonic Conditions	Biofilm Conditions	Planktonic Conditions	Biofilm Conditions	Planktonic Conditions	Biofilm Conditions
Synergistic (FICI≤0.5)	17	18	17	18	17	19
Additive (0.5<FICI ≤1)	17	17	18	17	17	16
No interaction (1<FICI<4)	5	4	4	4	5	4
Antagonistic (FICI≥4)	0	0	0	0	0	0
Sensitization rate	43.59%	46.15%	43.59%	46.15%%	43.59%	48.72%

Abbreviation: FICI, fractional inhibitory concentration index.

enhancement rates for the agents under biofilm conditions were 46.15%, 46.15%, and 48.72%, respectively.

Time–Growth Curves

C. albicans ZY23 was treated with FCA, ITR, or VRC alone or in combination with different concentrations of ASA to construct time–growth curves. As shown in Figure 1, 8 μg/mL FCA, 4 μg/mL ITR, or 4 μg/mL VRC inhibited the growth of *C. albicans* ZY23. Compared with the FCA alone group, FCA combined with ASA further inhibited the growth of ZY23, and ASA at a concentration of 8 mg/mL showed better synergistic inhibitory effects than it did at 4 mg/mL (Figure 1A).

There was no significant difference in ZY23 growth among the ITR alone, ITR combined with 1 mg/mL ASA, and ITR combined with 2 mg/mL ASA groups. ITR

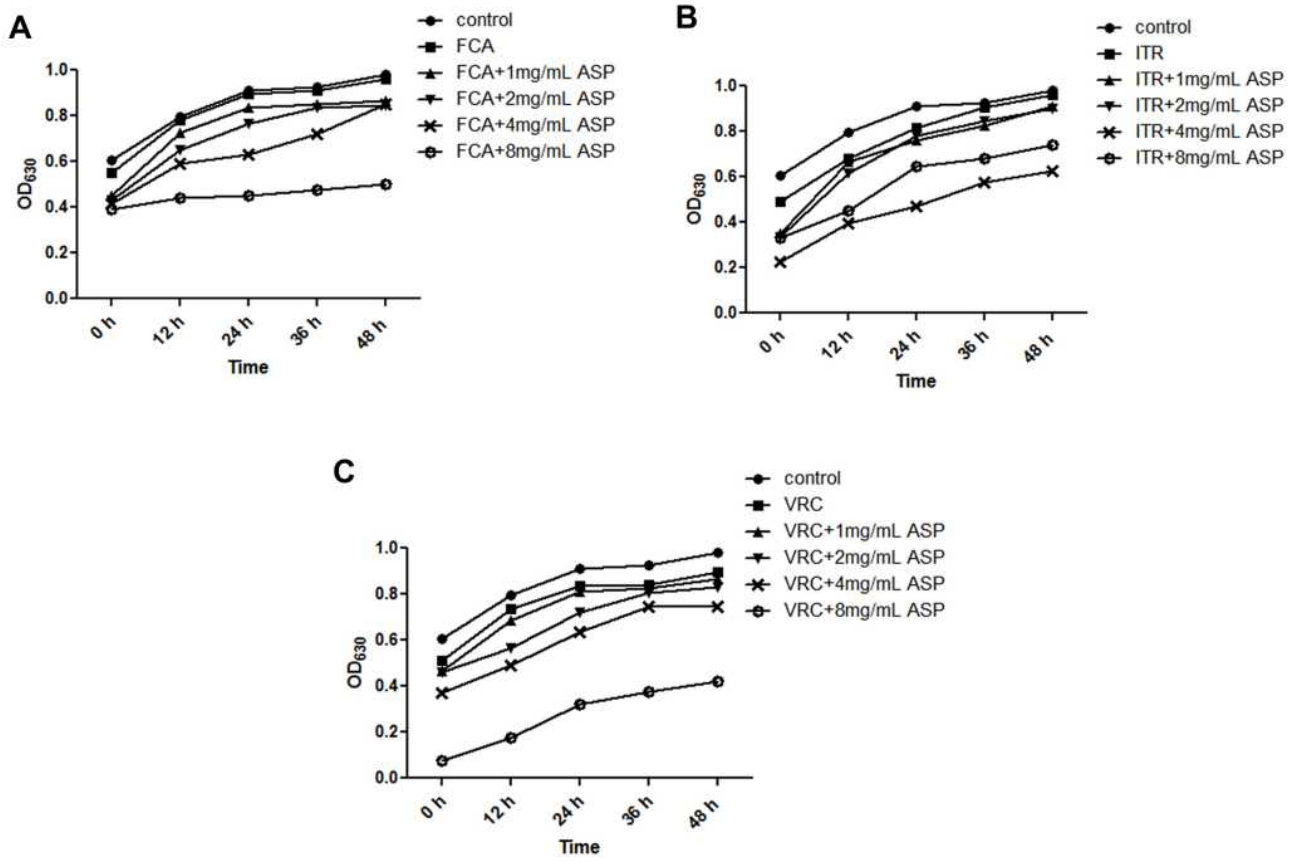

Figure 1 Time–growth curves of *Candida albicans* ZY23. Time–growth curves of ZY23 treated with (**A**) fluconazole (FCA) (**B**) itraconazole (ITR) or (**C**) voriconazole (VRC) alone or in combination with different concentrations of aspirin (ASA).

combined with 4 mg/mL or 8 mg/mL ASA suppressed the growth of ZY23 more than it had done alone, and showed better effects in combination with 4 mg/mL ASA (Figure 1B). Furthermore, treatment with VRC alone or in combination showed a similar trend to that with FCA or ITR. VRC in combination with 8 mg/mL ASA showed obviously better inhibitory effects on *C. albicans* than it had done alone (Figure 1C). Based on these results, 8 μg/mL FCA combined with 8 mg/mL ASA, 4 μg/mL ITR combined with 4 mg/mL ASA, and 4 μg/mL VRC combined with 8 mg/mL VPL were chosen for the subsequent time-kill curve experiments.

Time-Kill Curves

After drug treatment, the total number of colonies at each time point was calculated to assess the synergistic inhibitory effect of the drugs on the *C. albicans* strain. After a 2 h culture, the growth of ZY23 was significantly inhibited by FCA alone and in combination with ASA, whereas the inhibitory effect of FCA alone on *C. albicans* was not obvious after a 24, 36, and 48 h culture compared to the control treatment. Compared with the FCA alone group, the number of *C. albicans* colonies in the FCA plus ASA group was lower, suggesting that the combination could have better inhibitory effects on the growth of *C. albicans* than FCA alone (Figure 2A). The inhibitory effect of ITR or VRC alone or in combination was similar to that of FCA alone or in combination (Figure 2B and C). These results indicated that azole drugs may have more significant inhibitory effects on *C. albicans* in combination with ASA than they do alone.

Discussion

Recently, drug combinations have become an effective strategy to overcome the increasing resistance of *C. albicans* and other pathogenic diseases causing organisms. ASA is mainly used for its antipyretic, analgesic, and anti-inflammatory activities. Previous studies have shown that ASA increased the sensitivity of FCA-resistant strains and inhibited the growth of *C. albicans* under planktonic conditions.[26,27] In our experiment under planktonic conditions, the MIC_{50} values of FCA, ITR, or VRC applied alone were 64–0.5 μg/mL, 32–0.0625 μg/mL, and 16–0.125 μg/mL, respectively, whereas cotreatment with ASA decreased the values to 32–0.25 μg/mL, 8–0.0313 μg/mL and 8–0.0313 μg/mL, respectively. Additionally, the rate of enhancement of the inhibitory activities of FCA, ITR, and FCA by ASA under planktonic conditions

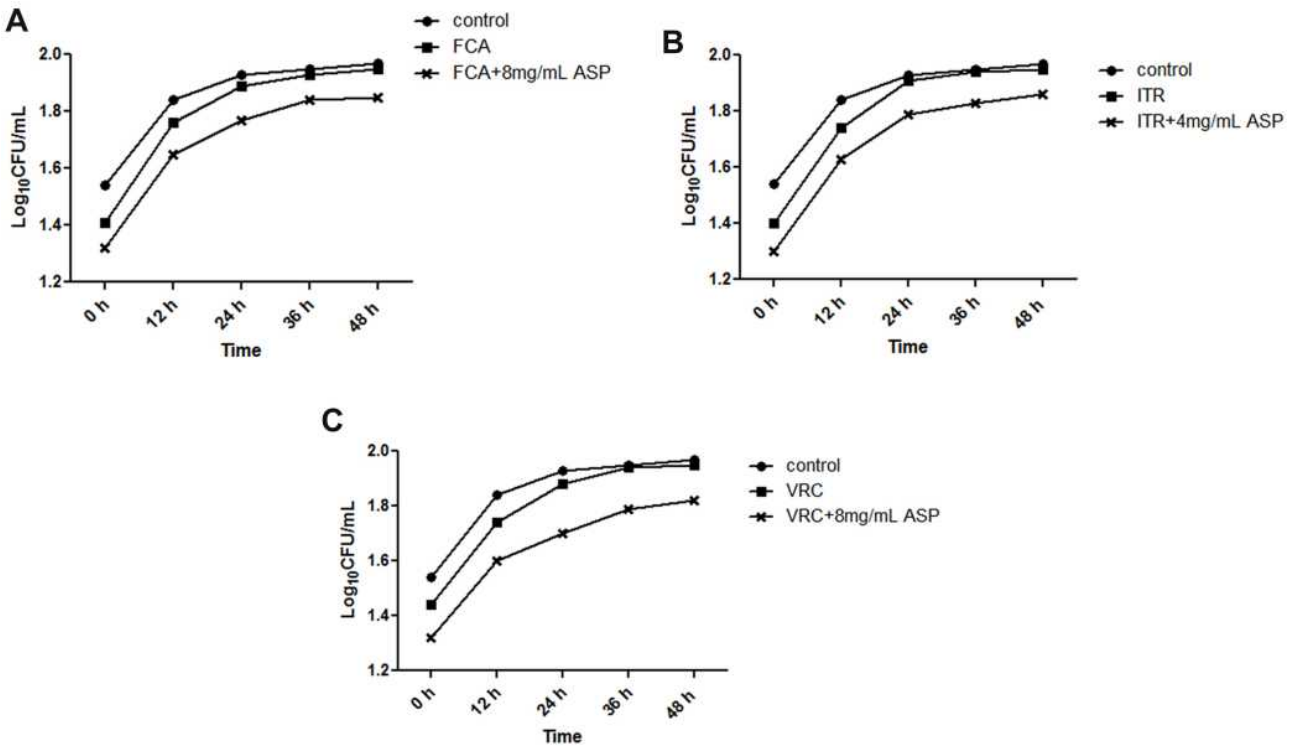

Figure 2 Time-kill curves of *Candida albicans* ZY23 treated with (**A**) fluconazole (FCA) (**B**) itraconazole (ITR), and (**C**) voriconazole (VRC) alone or combination.

was 43.59%. In addition, the results of the time-growth and time-kill curve assays also showed that compared to treatment with each azole drug alone, cotreatment with ASA further enhanced the growth inhibition of *C. albicans* ZY23. A previous study by Pina-Vaz et al[28] showed that cotreatment with ibuprofen and FCA showed synergic activity against 8 of the 12 *Candida* strains tested, including four of the five FCA-resistant strains, indicating that ibuprofen increased their sensitivity. Another study demonstrated that the cotreatment with FCA and ibuprofen, propylparaben, or sodium salicylate resulted in a synergistic activity with an FICI < 0.5 against *C. albicans* NCYC610.[29] Feng et al[24] also reported that ASA and verapamil increased the sensitivity of *C. albicans* to caspofungin under planktonic conditions, and may be a sensitizer for caspofungin against *C. albicans* under planktonic conditions. Our results corroborate these findings and led to the inference that ASA could increase the sensitivity of clinical *C. albicans* and may be used to sensitize this fungus to azole drugs under planktonic conditions.

C. albicans usually exist in the body under two states (planktonic and biofilm). Biofilm is an extracellular polymeric matrix produced by the microbial community for self-protection that can adhere to the surface of living or non-living materials.[30] The biofilm state, a survival mode that differs from the planktonic state, is the organism that adopts to adapt to long-term environmental pressure.[31] Mature biofilms are a dense mesh system consisting of yeast cells, hyphae, and pseudohyphae surrounded by numerous extracellular polymers.[32] Additionally, biofilm formation may be correlated with the antimicrobial resistance phenotype. Senobar Tahaei et al[33] investigated 300 strains of clinical *Staphylococcus aureus* and did not find any associations between methicillin resistance and biofilm production, whereas erythromycin, clindamycin, and rifampin resistance were associated with biofilm positivity. These findings indicate that biofilm formation may be a factor leading to *C. albicans* resistance. A previous study proposed NSAIDs as potential antimicrobials based on their inhibition of quorum sensing in the investigation of drug repurposing strategies for antibacterial and anti-virulence effects.[34]

ASA is an NSAID and to further elucidate its effects on *C. albicans* growth in combination with azole drugs, the biofilms of *C. albicans* were established and the drug MIC_{50} values were measured. In the current experiment under biofilm conditions, cotreatment with ASA decreased the MIC_{50} values of FCA, ITR, and VRC from 128 to 8 μg/mL to 32–0.5 μg/mL, 32–4 μg/mL to 16–0.5–μg/mL, and 32–0.5 μg/mL to 8–0.0625 μg/mL, respectively. Based on the FICI results, the rates at which ASA sensitized the fungal strain to FCA, ITR, or FCA under biofilm conditions were 46.15%, 46.15%, and 48.72%, respectively. Alem et al[35] reported that ASA dramatically inhibited biofilm formation in *C. albicans* GDH 2346 in a dose-dependent manner. At concentrations between 100 μM and 1 mM, ASA showed a > 70% inhibition of the biofilm, whereas at concentrations between 50 and 75 μM, the inhibition was only 20%, and 10 μM showed no effect. Zhou et al[36] explored the interaction between ASA and amphotericin B in their activity against *C. albicans* and *C. parapsilosis* under planktonic and biofilm conditions, and found that ASA enhanced the activity of amphotericin B, and the combination exhibited a strong synergistic effect on *C. albicans* and *C. parapsilosis* under biofilm conditions. Therefore, we speculated that cotreatment with ASA significantly reduced the MIC_{50} values of FCA, ITR, and VRC and synergized their inhibitory effects on *C. albicans* under biofilm conditions, thereby further suppressing the growth of *C. albicans* in the biofilm state.

Conclusion

In conclusion, ASA may serve as a sensitizer for azole drugs to further enhance their inhibition of the growth of clinical *C. albicans* under planktonic and biofilm conditions. However, the curative effects of the combination of ASA and azole drugs on *C. albicans* should be further verified in vivo, and the underlying mechanisms of action need to be further elucidated. Our findings provide a novel and potential therapeutic strategy for the clinical treatment of candidiasis and a theoretical basis for the use of ASA as a sensitizer for azole drugs in the treatment of *C. albicans* infection.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Disclosure

The authors report no conflicts of interest in this work.

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