In Vitro Interactions between Aspirin and Amphotericin B against Planktonic Cells and Biofilm Cells of C. albicans and C. parapsilosis

Yabin Zhou1Δ, Ganggang Wang1Δ, Yutang Li1, Yang Liu1, Yu Song1, Wenshuai Zheng1, Ning Zhang1, Xiaoyan Hu1, Shikun Yan1, Jihui Jia1*

1Department of Microbiology and Key Laboratory for Experimental Teratology of Chinese Ministry of Education, School of Medicine, Shandong University, Jinan, PR China.

Keywords: C. albicans, C. parapsilosis, Biofilm cell, checkerboard microdilution method, time-killing test, FICI, ΔE model

Δ These authors contributed equally to this work

*Corresponding author: Mailing address: Department of Microbiology, School of Medicine, Shandong University, Jinan, Shandong, 250012 P.R. China. Phone: 86- 531-88382672. Fax: +86 531 88382502. E-mail: jiajihui@sdu.edu.cn
ABSTRACT:
The increase of drug resistance and invasion caused by biofilm formation brings enormous challenges to the management of Candida infection. Aspirin’s antibiofilm activity in vitro was found recently. The spectrophotometric method and XTT reduction assay used for data generation make it possible to evaluate fungal biofilm growth accurately. The combined use of the most commonly used methods, the fractional inhibitory concentration index (FICI) and a newly developed method, the \( \Delta E \) model, which uses the concentration-effect relationship over the whole concentration range instead of using the MIC index alone enables the interpretation of results more reliable. As an attractive tool for studying the pharmacodynamics of antimicrobial agents, time–kill curves can provide detailed information about antimicrobial efficacy as a function of both time and concentration. In the present study, in vitro interactions between aspirin (ASA) and Amphotericin B (AMB) against planktonic cells and biofilm cells of C. albicans and C. parapsilosis were evaluated by the checkerboard microdilution method and time-killing test. Synergistic and indifferent effects were found for the combination of ASA and AMB against planktonic cells, while strong synergy was found against biofilm cells analyzed by FICI. The \( \Delta E \) model gave more consistent results with FICI. The positive interactions in concentration were also confirmed by the time-killing test. What’s more, it also revealed the pharmacodynamics changes of ASA and synergistic action on time. Our findings suggest a potential clinical use for combination therapy with ASA and AMB to augment activity against biofilm-associated infections.
INTRODUCTION

Infections caused by Candida species manifest in a number of diseases, including candidemia, vulvovaginal candidiasis, endocarditis, and peritonitis. Candida albicans and Candida parapsilosis are still two of the leading Candida species causing infections worldwide [45].

Microbial biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances and exhibit an altered phenotype with respect to growth rate and gene transcription [12]. Biofilms are ubiquitous in nature and are characterized by their recalcitrance towards antimicrobial treatment [42]. They are notoriously resistant to antimicrobial agents of various types, including biocides, antibiotics, and antiseptics [17].

Drug resistance has been demonstrated for Candida biofilms growing on surfaces such as cellulose, polystyrene and denture acrylic, as well as polyvinyl chloride [35, 3]. Therefore, there is a continuous need for the discovery of new antimicrobial agents that are effective against biofilms.

Among these efforts, one aspect is to explore 'nonantibiotic drugs' for their antimicrobial and antibiofilm activities. The previous results show that aspirin, one of the oldest and most widely used anti-inflammatory drugs, dramatically decreases biofilm formation by C. albicans. Alem et al found aspirin was active against growing and fully mature (48-h) biofilms and its effect was dose related [3].

Aspirin is known to have a variety of effects on microorganisms. In vitro studies on planktonic cultures demonstrated its antifungal [41], antibacterial [47] and antiviral [23] activities. These effects include changes in membrane potentials and production of virulence factors, reduction in extracellular polysaccharide and prostaglandin production [41; 3], which may contribute to aspirin’s antimicrobial activities against planktonic and biofilm cultures. Although some aspirin concentrations (50 to 200 μM) producing significant levels of antibiofilm activity in vitro fall within the range of those frequently achieved by therapeutic doses of aspirin in humans [48]. The significant effects of aspirin on growth and biofilm formation of Candida spp. were achieved only with suprapharmacological concentrations of the drug, which limits its clinical application [41].

Amphotericin B (AMB), a polyene macrolide agent, used as “the gold standard” antifungal drug since 1960s, is a crucial agent in the management of serious systemic fungal infections. In spite of its proven track record, its well-known side effects and toxicity will sometimes require discontinuation of therapy despite a life-threatening systemic fungal infection. Conventional amphotericin B, despite being a broad-spectrum fungicidal agent with little intrinsic or acquired resistance, is limited by its serious toxicities and lack of an oral formulation for systemic therapy. Although improvements in manufacturing over the last 40 years have enhanced tolerability, infusion-related reactions and renal dysfunction are still commonplace with the use of the deoxycholate solubilized formulation, which still imposes restrictions on its treatment in comparison to newer triazoles and echinocandins.

What’s more, the high prices of amphotericin B’s lipid complex limit its large-scale application [22, 6, 21].

Combination therapy is one approach that can be used to improve the efficacy of antimicrobial therapy for difficult-to-treat infections [1]. Attempts have been made to cope with treatment failures either by combining different antifungals or by combining antifungals with nonantifungals [1, 26, 40]. Aspirin, which possesses a weak and broad-spectrum antimicrobial activity towards some planktonic and biofilm cultures, may be useful in combined therapy with conventional antifungal agents to reduce their dose and improve their efficacy, while amphotericin B, needs to be combined with agents that are cheaper, more effective, more tolerable, and less toxic, particularly less nephrotoxic than AMB deoxycholate. Considering these, combination between aspirin and
amphotericin B is an excellent choice for clinical use. In the present study, we investigated the minimal inhibitory concentration (MIC) and combined effects of aspirin and amphotericin B against the planktonic cells and biofilm cells of C. albicans and C. parapsilosis by microdilution method and the checkerboard microdilution method, and compared the differences of drug efficacy in different states. Furthermore, we obtained the detailed information about antimicrobial efficacy as a function of both time and concentration by time-killing test to assess the pharmacodynamics of each antimicrobial agent and the combined effects. There have been controversies over assessing the nature and intensity of drug interactions and the observed in vitro interaction of two agents depends on different methodology for data generation and analysis, resulting in variable as well as controversial conclusions [33]. New methods and interpretation models such as ΔE model were employed in comparison with FICI. To improve the accuracy of results and reproducibility, we utilized spectrophotometric method and colorimetric method instead of the traditional methods of visual reading and colony counting.

MATERIAL AND METHODS

Candida strains Overnight cultures of the following microorganisms were used throughout the study: each one of the strains of C. albicans CCA 10, C. albicans YEM30 and C. parapsilosis ATCC 22019. Long-term maintenance of the microbial strains was at −20°C using glycerol and short-term maintenance was on nutrient agar plates at 4°C. CCA 10 was isolated from a patient with invasive candidiasis from QiLu hospital and was confirmed according to standard mycological methods by the Microbiological Research Laboratory, the Center of Health Research and Epidemic Prevention, Shandong Province. C. albicans YEM30 was obtained from the Key Laboratory of Oral Biomedicine of Shandong Province. C. parapsilosis ATCC 22019 was obtained from the Key Laboratory for Experimental Teratology of Chinese Ministry of Education. All strains were maintained on slopes of Sabouraud dextrose agar (Difco) and subcultured monthly. Every 2 months, cultures were replaced by new ones freshly grown from freeze dried stocks.

Candida spp. suspension Prior to each experiment, the yeast strain was cultured aerobically at 30°C for 18 h on YPD medium containing 1% (w/v) yeast extract, 1% (w/v) bacto peptone and 2% (w/v) glucose. The strains were cultured in the budding-yeast phase under these conditions. After 18 h of incubation, the cells, which were in the late exponential growth phase, were harvested, washed three times with PBS (pH 7.2) and resuspended in YPD medium. The concentrations of the Candida suspensions were measured by blood cell count plate.

Aspirin and amphotericin B solutions Stock solution of amphotericin B (NCPC, H13020284; 5mg/ml) was freshly prepared in phosphate buffer saline (PBS) that was sterilized by autoclaving. Stock solution of aspirin (Sigma; 900 mg/ml) was freshly prepared in dimethylsulfoxide (DMSO), then filtration was performed for sterilization. In experiments performed on planktonic cells and biofilm cells, aspirin was used at final concentrations ranging from 0.03125 mg/ml to 16 mg/ml, and amphotericin B was used at a final concentration ranging from 0.015625μg/ml to 64μg/ml.

Minimal inhibitory concentration (MIC) MIC values of aspirin and AMB were studied by the broth microdilution method in 96-well plates according to the CLSI standard M27-A2, using RPMI 1640 medium (buffered to pH 7.0±0.1 with MOPS buffer) [30]. For the MIC studies, stock solutions of 160mg/ml of aspirin and 256μg ml⁻¹ of AMB were used. Each drug was first serially diluted 2-fold. A volume of 100μl of either
aspirin or AMB was added. Each suspension (5 \times 10^6 CFU/ml) was diluted (1:50 dilution followed by 1:20 dilution) in RPMI 1640 to obtain two times the final inoculum size of 2.5 \times 10^7 CFU/ml, then 100 \mu l inoculum was added. The plates were incubated for 48 h at 37°C, MIC was expressed as minimal concentration of the well showing 20% growth for planktonic cells (50% for biofilm cells) comparing to positive control wells. Growth was detected as turbidity (492nm) relative to an uninoculated well using a microtitre plate reader. Negative controls were performed with only RPMI1640 in each well, and positive controls were performed with only microorganisms overnight culture in the wells. Positive control included DMSO in the same concentration (v/v) as that used in the experimental substances. Each MIC determination was performed in triplicate.

**Measure of antimicrobial combination** The in vitro interaction between aspirin and AMB was studied by a two-dimensional (eight by twelve) checkerboard microdilution technique in sterile, 96-well flat-bottom microtitration plates as described below. Each isolate was tested three times on different days. The concentration of each antimicrobial agent in combination ranged from 1/32 times to four times the MIC. For the combination studies, each drug was first serially diluted 2-fold in the corresponding solvents according to the MIC in order to obtain four times the final concentration. A 50\mu l aliquot of each drug concentration of the AMB was added to columns 1 to 10. Then, a 50\mu l aliquot of each concentration of aspirin was added to rows A to G. In the wells of column 10, 50 \mu l of the medium containing aspirin solvent was added, and in wells of row H, 50 \mu l of the medium containing AMB solvent was added. Thus, row H and column 11 contained only AMB and aspirin, respectively, column 12 and the well at the intersection of row H and column 12 (well H12) was the drug-free well that served as the growth control. The final concentrations of aspirin and AMB were confirmed according to MICs. Each well was inoculated with a final inoculum size of 2.5 \times 10^3 CFU ml^{-1} of the tested microorganisms. The setup was incubated at 37°C for 48 h. Then, a microtitre plate reader was used to detect growth (growth) as turbidity (492nm) relative to an uninoculated well.

**Candida biofilm development** Candida biofilm formation was performed as described by Wander et al [10]. Briefly, aliquots of 100 \muL of standard cell suspensions of yeasts (10^7 cells/ml, in YPD medium) were transferred into each well of 96-well microtiter plates and incubated for 1.5 h (adhesion phase) at 37°C at 75 rpm in an orbital shaker. After the adhesion phase, the cell suspensions were gently aspirated and each well was washed twice with PBS to remove any remaining planktonic. Adhered cells remained undisturbed during the whole process. In order to allow the growth of biofilm (biofilm phase), 200 \muL of freshly prepared YPD medium was added to each well. The plates were incubated for 24h, 48h at 37°C at 75 rpm in an orbital shaker. At 24 h of incubation, the medium was aspirated and biofilms were washed twice with PBS followed by addition of 200 \muL of fresh medium.

**Drug addition of biofilm susceptibility testing** At 48h of incubation (maturation of the biofilm), the medium was aspirated and biofilms were washed twice with PBS followed by addition of 100 \muL of fresh medium at first. Then, each serially diluted drug was added. For the MIC studies, a volume of 100 \muL of either aspirin or AMB was added. For the combination studies, checkerboard assay was used to evaluate the antimicrobial efficacy of aspirin and AMB (ASA-AMB) upon combination as above. We chose the appropriate range of drug concentrations for the combination studies according to the biofilm MICs of the individual drugs we just got, which was a lot different from those of planktonic cells. The setup was incubated at 37°C, 75 rpm in an orbital shaker for another 24h. Then, the biofilms would be quantified using the XTT reduction assay. All assays were repeated 3 times in two separate occasions.

**Oxidative activity assay** Quantitation of Candida biofilms was performed as described previously [10, 20] by
using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-\((\text{phenylamino})\text{carbonyl}\)-2H-tetrazolium hydroxide (XTT; Sigma-Aldrich Corp.) reduction assay. XTT was reduced by mitochondrial dehydrogenase into a water-soluble formazan product that was measured spectrophotometrically. XTT was dissolved in PBS (without glucose) at a final concentration of 1 mg/mL. The solution was filter-sterilized and stored frozen at -70°C until use. Menadione solution (0.4 mM; Sigma-Aldrich Corp.) was prepared immediately before each assay. Anhydrous D (+) glucose was dissolved in sterile PBS to obtain PBS with 200 mM glucose. For each assay, XTT solution was thawed on ice and mixed with menadione solution at a volume ratio of 20:1. After washed twice with 200 µL of PBS to remove nonadherent cells, 158 µL of PBS with 200 mM glucose, 40 µL of XTT and 2 µL of menadione were transferred to each well of 96-well plates, then the plates were covered with aluminum foil and incubated in dark at 37°C for 3 h. Thereafter, 100 µL of the solution was transferred to each well of new 96-well plates. The colorimetric changes were measured at 492 nm using a microtiter plate reader.

**Time-kill curve (TKC)** To make a further investigation on the effect of concentration and exposure time on the drug activities and assess the pharmacodynamics of each antimicrobial agent and the combines agents, we performed a time-kill test in vitro to investigate the activity of serially diluted aspirin combined with and without amphotericin B against the biofilm of CCA 10, YEM30 and ATCC 22019. This methodology was first used in planktonic cells by Ernst et al [13]. We utilized it to study biofilm cells with some modifications. Briefly, the Albicans suspension in YPD medium (10^7 cells/ml) was prepared and mature biofilms in 96-well microtiter plates were developed as described previously. At 48h of incubation, the medium was aspirated and biofilms were washed twice with PBS, followed by addition of 100 µL of fresh medium. Aspirin was used at concentrations of 0.25, 0.5, 1, 2.0, 4.0 mg/ml for CCA 10, ATCC22019 and 0.5, 2.0, 4.0, 8.0 mg/ml for YEM30. Amphotericin B was used at 8.0 µg/ml for the above strains (16µg/ml, 32 µg/ml was also used for YEM30). Time-kill studies (for example, CCA10) were conducted on twelve groups: (1) control-no drugs added, (2) ASA 0.25, (3) ASA 0.5, (4) ASA 1, (5) ASA 2, (6) ASA 4, (7) AMB 8, (8) AMB 8 + ASA 0.25, (9) AMB 8 + ASA 0.5, (10) AMB 8 + ASA 1, (11) AMB 8 + ASA 2, (12) AMB 8 + ASA 4.) Microtiter plates covered with aluminum foil were placed on an orbital shaker (75 rpm) and incubated at 37°C during the experimental process. At predetermined time points (3, 6, 9, 12, 18, 24, 36 and 48 h), the media of the corresponding wells were aspirated and washed twice with PBS. Then, 200µl aliquots of XTT-menadione solution were added. After 3h incubation, 100 µL of the XTT solution was transferred to corresponding well of new 96-well plates. Thereafter, the colorimetric changes were measured at 492 nm by microtiter plate reader.

**Drug interaction modeling** In order to assess the nature of the in vitro interactions between aspirin and amphotericin B against each Candida strain, the data obtained by the spectrophotometric method were analyzed using two different models, FICI and ΔE, that have been used to characterize antifungal drug interactions[24,44,40]. Among different models and approaches, the assumption of no interaction has a central position, since synergy and antagonism are defined as departures from this. In the Loewe additivity (LA) -based models, concentrations of the drugs, alone or in combination, that produce the same effect are compared. In the Bliss independence (BI)-based models the estimates of the combined effect based on the effect of the individual drugs were compared with these obtained by the experiment. FICI and ΔE are nonparametric models based on LA theory and BI theory respectively.

**LA-based models (FICI)** The nonparametric approach is based on the fractional inhibitory concentration (FIC) index model expressed as  \( \sum \text{FIC} = \frac{\text{FIC}_A + \text{FIC}_B}{\text{MIC}_A^{\text{comb}} / \text{MIC}_A^{\text{alone}} + \text{MIC}_B^{\text{comb}} / \text{MIC}_B^{\text{alone}}} \) (equation 1.1), where
MIC\(_A\)^{alone}\) and MIC\(_B^{alone}\) are the MICs of the drugs A and B when acting alone and C\(_A^{comb}\) and C\(_B^{comb}\) are concentrations of the drugs A and B at the iso-effective combinations, respectively. Among all \(\Sigma FIC\)s calculated for each data set, the FIC index was determined as the \(\Sigma FIC_{min}\) (the lowest \(\Sigma FIC\)) when the \(\Sigma FIC_{max}\) (the highest \(\Sigma FIC\)) was less than 4; otherwise, the FIC index was determined as the \(\Sigma FIC_{max}\). Two MIC endpoints were used for the evaluation of each data set. The MIC-1 (MIC20) and MIC-2 (MIC50) were defined as the lowest drug concentrations of the drugs A and B at the iso-effective combinations, respectively. Off-scale MICs were converted to the next-highest or -lowest doubling concentration. Finally, the median and the range of FIC indices of the replicates were determined for MIC-1 and MIC-2. According to the work of Odds [17], for the interpretation of a single FICI, a value \(\geq 4\) is usually considered antagonism, and a value \(\leq 0.5\) is synergy, in all other cases indifference is concluded. In this study we interpreted the results according to the definition of Odds. In addition, isobolograms were plotted in order to visualize the departure from additivity [31].

**BI-based models (\(\Delta E\) model)** The BI theory is described by the equation
\[
\begin{align*}
I_i &= I_A + I_B - I_A \times I_B \\
I_A, I_B &= \text{the experimental percentages of inhibition of each drug acting alone, respectively. Since} \\
E &= 1 - E_i \\
E_i &= \text{the predicted percentage of growth of the theoretical noninteractive combination of the drugs A and B, respectively, and} \\
E_A, E_B &= \text{the experimental percentages of growth of each drug acting alone, respectively. Interaction is described by the difference (\(\Delta E\)) between the predicted and measured percentages of growth with drugs at various concentrations (\(\Delta E = \Delta E_{predicted} - \Delta E_{measured}\)). Because of the nature of interaction testing using microtiter plates with twofold dilution of either drug, this results in a \(\Delta E\) for each drug combination. A 3-D plot with the \(\Delta E\) depicted on the z axis results in a surface plot.}
\end{align*}
\]

For each combination of the two drugs in each of the three independent experiments, the observed percentage of growth obtained from the experimental data was subtracted from the predicted percentage, calculated as described above for each model. When the average difference was positive and its 95% CI among the three replicates did not include 0, synergy was claimed; when the difference was negative and its 95% CI did not include 0, antagonism was claimed. In any other case, BI was concluded. The values thus obtained for each combination were used to construct a 3-D plot. Peaks above and below the 0 plane indicate synergistic and antagonistic combinations, respectively, while the 0 plane indicates the absence of SS interaction (Fig. 2). The contour plots were also constructed in order to visualize the drug concentrations producing an interaction.

Since 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 synergistic (\(\Sigma SYN\)) and antagonistic (\(\Sigma ANT\)) interactions. Interactions with <100% of interactions were considered weak, those with 100 to 200% of interactions were considered moderate, and those with 200% of interactions were considered strong. In addition, the numbers of synergistic and antagonistic combinations among all the combinations tested were calculated for each strain.

**RESULTS**

**Drug susceptibility alone and in combination** CCA 10, YEM30 and ATCC 22019 were selected in this study. The MICs of two control strains, ATCC 22019 and ATCC 6528, were within the normal range. The results from the testing drug alone and in combination against the 3 tested microbial strains are summarized in Table 1. By
testing the drug alone, in planktonic cells, aspirin has weak effect on the tested strains and AMB has strong fungicidal effect, while in biofilm cells, the highest level of resistance to AMB is observed, with the MIC-2 (IC50s) to the corresponding strain increased up to 64- and 128-fold after biofilm formation, respectively, based on MICs by XTT. However, aspirin’s fungistatic activity in biofilm cells seems to change little in comparison to planktonic cells, which is consistent with the previous report(s) and indicates dramatic antibiofilm activity. When combined with AMB, a potent fungistatic effect was revealed, especially in biofilm cells. In terms of planktonic cells, the MICs of either individual agent were reduced by one to two dilutions against the tested strains, while remarked reductions were observed for AMB against biofilm cells when combined with aspirin. The geometric mean (GM) of the MIC-2 to AMB and aspirin decreased up to 32- and 16-fold, respectively, based on FIC indices. The MIC-2 of AMB and aspirin to tested stains were then shifted down to 1–2μg/ml and 0.031–0.063 mg/ml, respectively. However, the MICs of AMB against biofilm cells were still a little higher than those against planktonic cells. The percentages of fungal growth for each combination were calculated by comparing the OD of the drug-containing well with that of the drug-free well after subtracting the background OD obtained from the microorganism-free well. The interactions between AMB and aspirin against biofilm cells are presented in Fig. 1, 2, 3.

**Interpretation of drug interactions** The results of the checkerboard analysis interpreted by the nonparametric methods based on both the LA and BI theories are summarized in Table 2. In the checkerboard microtiter format, strong synergism was observed in biofilm cells of all three stains analyzed by FICI and ΔE. The two models correlated very well. The FICI indexes were far less than 0.5, while all the ΣSYNs of the three tested strains were beyond 900%, which indicate a super strong synergistic action between aspirin and amphotericin B. As to planktonic cells, synergisms were observed in ATCC22019, the FICI index is 0.5, the average ΔE was positive and its 95% CI among three replicates did not include 0 and ΣSYN >200%, both of them show a strong synergistic action. Indifference was observed in CCA10, the FICI index is 1.0, the 95% CI of the average ΔE included 0, both of them revealed indifferent action. However, the interpretations to ATCC22019 by two models seemed to be a little different. According to the definition of Odds, indifference was observed by FICI, while in ΔE model the average ΔE was positive and its 95% CI among the three replicates did not include 0 and ΣSYN >100%, which revealed a moderate synergistic action (Table 2). However, according to another standard, the FIC indices of YEM30 in all three replicates were less than 1, synergy can also be claimed. From that point, we also obtained consistent results by the two models. In addition, the values obtained for each combination based on BI theory were used to construct a three-dimensional plot. Thus, a surface plot was obtained by using a three-dimensional plot, with ΔE depicted on the z axis. The contour plot was also constructed in order to visualize the drug concentrations that produce an interaction (Fig. 1, 2, 3).

**Time-killing curves** The concentration-dependent synergistic action of aspirin and amphotericin B against biofilm cells that proved by checkerboard microdilution assay was confirmed by time-kill curves. Furthermore, the curves also revealed the time-dependent synergistic action between them. The time-kill curves, cell viability (OD) versus time, were plotted and used for visual comparisons of the rate and extent of antifungal activity for aspirin and combination. The intensity and nature of interactions between aspirin and AMB against YEM30, ATCC22019 and CCA10 are also shown in Fig.4. In aspirin alone, although the rate of killing tested strains was gradual, it is heterogeneously changing versus time. And discernible improvement in the extent of fungistatic activity was noted as the amount of drug in solution was increased. Similarly, the rate of activity for each stains improved and there was a trend toward a shorter time to the fungistatic endpoint as the concentration of aspirin in solution increased. In combination, the curves revealed that AMB itself can hardly affect the biofilm growth at 8μg/ml over time, but the
antibiofilm activity of aspirin against biofilm cells was dramatically enhanced by addition of it. The combination of AMB and aspirin also resulted in a faster rate of killing, measured by the time necessary to achieve a fungicidal endpoint and the slope function of the curves in Fig. 4 (AI, B, C). The slope of the curve for aspirin (4mg/ml) against YEM30 was not influenced after adding AMB (8μg/ml), however, the addition of AMB (16μg/ml, 32μg/ml) to aspirin (4mg/ml) enhanced its activity strikingly, which indicated the intensity of interactions varied according to drug doses. What’s more, in drug alone, increasing the concentration of AMB in small range displayed indifference in antifungal activity, while dramatical enhancement was found in combination with aspirin Fig. 4A (I, II, III). In summary, a strong concentration- and time-dependent synergistic action of ASA and AMB against Candida biofilms was found, and the most suitable therapeutical concentration of ASA and AMB for clinical use needs further optimization.

DISCUSSION

C. albicans biofilm is a heterogeneous community of yeast, pseudohyphal, and hyphal cells embedded in an extracellular polymeric matrix, noticeably including soluble β-glucans [28]. The formation of biofilms is intimately associated with C. albicans pathogenesis [34]. C. albicans biofilms formed under clinical conditions display intrinsic resistance to antifungals. Consistent with these observations, a number of studies show that C. albicans biofilms obtained in vitro display decreased sensitivity to almost all available antifungals: amphotericin B (AMB), flucytosine, terbinafine, nystatin, and, most notably, azoles [18]. Fifty percent inhibitory concentrations (IC50s) are increased as much as 10-fold, while MICs are increased 30- to 20,000-fold; the highest level of resistance observed is that to azoles [7, 9].

The formation of biofilms as a source for antimicrobial treatment failure requires the discovery and development of new antimicrobial therapeutic agents. However, the efforts of identification and exploitation of novel targets and development of the related inhibitors have proved to be frustrating and not financially rewarding for the pharmaceutical industry, so the discovery of new medicinal value of the old classic drug is highly significant [43].

Recently, however, it has been reported that aspirin dramatically decreases biofilm formation by C. albicans in vitro [48, 3]. Acetylsalicylic acid (ASA, aspirin) is a well-known nonsteroidal anti-inflammatory drug (NSAID) with analgesic, antipyretic and anti-inflammatory properties. ASA significantly decreases C. albicans biofilm formation and reduces viability of biofilm cells at concentrations that could be achieved in humans with therapeutic doses [3]. Additional studies found that ASA also suppresses biofilm formation of C. guilliermondii, C. kefyr, C. glabrata, and C. parapsilosis [41]. Thus it has been suggested to use in combination with conventional antifungal agents in the management of biofilm-associated Candida infections [3].

As a nonselective COX inhibitor, ASA inhibits COX-1 by hindering active site accessibility by acetylating the serine-530 residue. Various COX inhibitors decreased biofilm formation by C. albicans, with aspirin producing the greatest effects [3]. Biofilms of C. albicans produced much more prostaglandin than those of planktonic cells. In vivo, prostaglandin could significantly enhance fungal colonization and pathogenesis. The strong correlation between decreased prostaglandin level and decreased biofilm formation following exposure to COX inhibitors, support the notion that COX-dependent synthesis of prostaglandin may play a role in regulating biofilm development. However, the exact role of COX inhibitors and prostaglandin in fungal morphogenesis and biofilm development is obviously complex and remains unclear [4]. Aspirin has also been verified to suppress
morphogenesis of C. albicans hyphae and filamentous structures by inhibiting the formation of 3(R)-hydroxyxylophilins, oxygenated fatty acid metabolites derived from arachidonic acid [11]. What’s more, ASA may acetylate the active serine residue of the catalytic triad of extracellular secreted lipase active site that prevents the nucleophilic residue from properly interacting with substrate, thus reduces damage to reconstituted human tissues infected with Candida species [46].

Clearly, it would be of interest to investigate combinations of antifungal agents and aspirin in Candida biofilm assays, with a view to their possible use in combined therapy for the management of some biofilm-associated infections. However, growth of certain bacteria in the presence of salicylate can induce multiple resistances to antibiotics. Paradoxically, it can also reduce resistance to some antibiotics. The activities of antifungal agents can also be affected by salicylate. A combination of fluconazole with either sodium salicylate or ibuprofen results in synergistic activity against C. albicans planktonic cells [32, 39]. Aspirin–EDTA combination has also shown synergistic activity against C. albicans biofilms and the exposure to the A-EDTA combination (4 h) resulted in complete bacterial biofilm eradication [2]. However, no results demonstrate that aspirin could be useful in combined therapy with conventional antifungal agents (such as Amphotericin B) in the management of some biofilm-associated Candida infections in detail and whether the combination activity is synergistic or antagonistic.

It is still unclear how aspirin can be used in related clinical application. In this study we applied checkerboard and time-killing curve experiments to Candida study through XTT reduction assay. We evaluated comprehensive interactions between aspirin and amphotericin B against planktonic cells and biofilm cells of ATCC 22019, YEM30 and newly clinically isolated C. albicans strains CCA10 by different methods and models. XTT reduction assay is capable of detecting differences in the rate and extent of antifungal activity and has been widely used in recent years [14, 8]. XTT is a new yellow tetrazolium salt which can be converted to a colored formazan in the presence of metabolic activity. Since the formazan product is water soluble, it is easily measured in cellular supernatants, which is important in biofilm research because it allows the study of intact biofilms, as well as examination of biofilm drug susceptibility without disruption of biofilm structure [8]. So as an indicator substance, XTT makes it accessible to evaluate fungal biofilm growth. The interactions were evaluated with the checkerboard microdilution assay based on antifungal growth, MIC50 was used as an endpoint for biofilm cells while MIC20 was used for planktonic cells. The results were interpreted by two nonparametric models, FICI, based on LA, and ΔE, based on BI no-interaction theories.

As we know, different results may be obtained by different methods. In recent years, considerable progress has been made in the methods of evaluating drug interactions. Spectrophotometric has been developed in recent years. They can quantify the fungal growth more precisely and are able to detect small changes in metabolic activity of fungi [25]. Based on these methods, the effects of antifungal drugs either on the fungal biomass or on the metabolic status of fungi can be measured. FICI is the most frequently used model to interpret the interaction between antifungal drugs. Ease of use, simplicity and feasibility of performance make FICI still the method of choice for analyses of drug-drug interactions. However, assessing the nature of drug interactions using the FIC index model presents several other problems besides the choice of the FIC indices and MIC endpoints, such as the lack of a good summary and statistical interpretation of the results and the imprecise approximation of the real FIC index when off-scale MICs are present. So the ΔE model, as a nonparametric method based on BI theory, is also developed as a useful approach in analyzing the nature of interactions between different drugs based on a checkerboard method. The model does not require the choice of the FIC indices and MIC endpoints to obtain statistical measures and is less sensitive to intraexperimental errors than the FIC index model. When they were...
used in assessing the interaction between aspirin and amphotericin B in our study, the percentages of fungal growth
were derived from the experimental data directly, and good agreement was found with the FICI model, particularly
against biofilm cells. Therefore, it was sufficient to indicate the strong synergistic action between aspirin and
amphotericin B to biofilms of C. albicans and C. parapsilosis.

Data collected from time-kill studies have provided critical information regarding the rate and extent of
bactericidal activity, pharmacodynamic characteristics (i.e., relationship between concentration and effect and the
postantibiotic effect), and potential antagonism or synergy between antibacterial agents administered
concomitantly. These data that provide growth kinetic information over time and give a more detailed picture of
the effect of drug combinations on cell viability have significantly enhanced our understanding regarding the
dynamic relationships which exist between antimicrobial agents and their effects on bacteria [26]. Our data from
time-kill curves based on metabolism-inhibitory effects confirmed the positive interactions observed in the
checkerboard test. The potency of all the tested aspirin can be enhanced by combining them with amphotericin B
against C. albicans and C. parapsilosis. In aspirin alone, discernible improvement in the extent of fungistatic
activity and the slope function of the time-kill curve to each strain was noted as the amount of drug in solution
increased. Marked concentration-dependent fungistatic activity was also observed, what’s more, the rate and extent
of fungistatic activity varied over time and the time to achieve a fungistatic endpoint was shortened as the dose
increased. In combination, the addition of amphotericin B in low dose to various concentrations of aspirin resulted
in strikingly improvement in extent of activity and trend toward a shorter time to the fungistatic endpoint versus
single agents. Thus, the combination therapy of amphotericin B and aspirin can enhance their rate and extent of
fungistatic activity and achieve maximal fungistatic activity in a shorter time in the case that both drugs alone
display weak fungistatic activity, which greatly enriched the meaning of combination therapy and produced
significant therapeutic effects at a low concentration (combination on concentration, reduce side effects and
improve efficacy) and in a short time (combination on time, reducing the time to fungistatic endpoint). Compared
with animal models of infection, in vitro models offer significant advantages in cost, convenience and time, as well
as permitting direct investigation on the antimicrobial interaction in a controlled and reproducible manner. The
time-kill testing can be an indispensable tool to pursuit the in vivo or in vitro study optimization of aspirin and
amphotericin B dosing regimens based the antifungal pharmacodynamic properties described for these agents [38].

Extensive research has focused on mechanisms of drug resistance in C. albicans biofilms. It is apparent that
cells in a fungal biofilm represent an epigenetic modification of the cellular state compared with their planktonic
counterparts, with changes in cellular morphology, cell-to-cell communication, and gene expression, as well as
with the production of an extra-cellular matrix [15, 36]. Multiple factors contribute to the elevated drug resistance
of C. albicans biofilms. These factors include increased cell density, increased expression of drug efflux pumps,
decreased ergosterol content, elevated β-1,3 glucan levels in the cell wall and biofilm matrix as well as signaling
mediated by protein kinase C (PKC) and the protein phosphatase calcineurin [36].

Pharmacological effects of aspirin to mature biofilm on specific aspects remain unclear. Previous studies have
shown that sodium salicylate inhibits biofilm formation and bacterial adhesion in a dose-dependent manner. It can
also inhibit the production of some components of ECM (such as polysaccharides, proteins and teichoic acid) by as
much as 95% [3]. C. albicans biofilms produce abundant extracellular polymeric matrix (ECM) during biofilm
growth under flow conditions which in turn increased resistance to antifungals [3, 5]. It has been shown that
facilitated by the amphiphilic nature, AMB binds to β-1,3-glucans, a structural component of the fungal cell wall,
as well as the biofilm ECM, alters β-1,3-glucans and increases the sensitivity of biofilms to AMB [3, 19]. Although high concentrations of AMB could be reached within the biofilm, entrapment of AMB in the ECM might prevent them from reaching biofilm cells. So despite accumulating in the biofilm at concentrations above the MIC, AMB are unable to kill biofilm cells completely [19]. One explanation of the synergistic activity is likely that aspirin inhibits the production of ECM, especially β-1, 3-glucans, and prevents them from binding with AMB, so high concentrations of AMB reach biofilm cells and exert its antifungal effect. Decreased levels of ergosterol, whose biosynthesis is inhibited by azoles and which is bound by AMB, and diminished expression of ergosterol biosynthetic genes have also been reported in mature C. albicans biofilms [19, 16]. It is interesting to investigate whether aspirin can disrupt the formation of mature biofilm and increase the content of ergosterol, which may increase the targets of amphotericin B and result in the synergistic activity. Exposure of C. albicans planktonic cells to azole antifungals is associated with the upregulation of genes that encode components of the ergosterol biosynthetic pathway and drug efflux pumps, thus providing a transient resistance mechanism [37]. Genes encoding drug efflux pumps, namely, MDR1, CDR1, and CDR2, are upregulated upon attachment of C. albicans cells to a surface, which accounts for the resistance of early-stage biofilms to azole drugs. Upregulation in CAS-exposed biofilms of several genes coding for cell wall proteins, in particular ALS3 and HWP1, plays important roles in biofilm formation [19, 27, 29, 49]. Thus, the investigation of aspirin to biofilm resistance related genes also helps explain the occurrence of synergistic activity. In addition, the increased activity shown by ASA-AMB combinations may also be explained by amphotericin B, which can create transmembrane channel and may therefore allow solutes such as aspirin to gain easier access and effect higher antimicrobial activity. What’s more, amphotericin B’s strong fungicidal effect can be very beneficial in counteracting the weak fungistatic activity of aspirin.

In summary, our work documented that the activity of amphotericin B can be enhanced by aspirin, a weak and broad-spectrum antimicrobial agent, with surprisingly potent antibiofilm activity in vitro. The results were confirmed by the normal MIC test with different interpretation models and the time-kill study to demonstrate the combined effects against Candida and it also suggested the different combination effects of the two drugs on planktonic cells and biofilm cells. Although a few assumptions have been offered here, further study is needed to determine the underlying mechanism of the synergistic action. However, this work leaves the door open for the clinical use of aspirin in combination with traditional antifungal drug (AMB) to treat infections based on biofilms in vivo, in vitro and in situ, especially for the superficial infections, where candidal biofilms colonizes several tissues, such as skin, vaginal and oral epithelia, or inert surfaces, such as dental prostheses. Yet, the possibility of this combination therapy in the management of systemic fungal infections warrants further investigation.

ACKNOWLEDGMENT

This work was supported by a grants from the Natural Science Foundation of Shandong Province, PR China. (Nos. ZR2009CM073, ZR2009CZ001, and ZR2009CM002), National Natural Science Foundation of China (Nos. 81171536, 30972775, 30971151 and 81170514) and the National Basic Research Program of China (973 Program 2012CB911202)

The analysis of drug interaction modeling was kindly guided by Qiongjie Guo (Department of Pharmacy, the First Hospital of Qinhuangdao, Qinhuangdao, 066000, R.P. China); Standard strain of C. albicans (YEM30) was kindly provided by Qingguo Qi (Department of Oral Medicine, the Key Laboratory of Oral Biomedicine of Shandong Province, R.P. China).
Reference


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<tr>
<th>Drug in combination, strain</th>
<th>Median MIC (range) of drug</th>
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<th></th>
<th></th>
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<td></td>
<td>ASA (mg/mL)</td>
<td>AMB (μg/mL)</td>
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<td>AMB (μg/mL)</td>
</tr>
<tr>
<td></td>
<td>F:MIC-1 (MIC80%)</td>
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<td></td>
</tr>
<tr>
<td>CCA10</td>
<td>2(1-2)</td>
<td>0.5(0.25-0.5)</td>
<td>1(0.5-1)</td>
<td>0.25(0.125-0.25)</td>
</tr>
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<td>2(0.5-2)</td>
<td>0.25(0.125-0.25)</td>
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</table>

αMICs of biofilm cells had little change in comparison to planktomic cells for aspirin alone, while the MICs to the corresponding strain increased dramatically for amphotericin B alone. When drugs were used in combination, for planktomic cells of the tested strains, MICs of planktomic cells showed no apparent changes based on the MIC-1, while for biofilm cells of the tested strains, MICs of aspirin and amphotericin B decreased markedly based on the MIC-2. Abbreviations: ASA, aspirin; AMB, amphotericin B; F, planktomic cells; BF, biofilm cells.
<table>
<thead>
<tr>
<th>Drug in combination, strain, and median</th>
<th>FICI Mean(range)</th>
<th>Interpretation</th>
<th>Nonparametric method&lt;sup&gt;a&lt;/sup&gt;</th>
<th>∆E model (%) Mean(range)</th>
<th>∑SYN(n)</th>
<th>∑ANT(n)</th>
<th>Interpretation</th>
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<td><strong>F:MIC-1 (MIC80%)</strong></td>
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<tr>
<td>CCA10</td>
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<td>SYN(S)</td>
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<td>SYN(SS)</td>
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<td>SYN</td>
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<td>0(0)</td>
<td>SYN(SS)</td>
<td></td>
</tr>
</tbody>
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<sup>a</sup>F, planktonic cells; BF, biofilm cells; SYN, synergism; ANT, antagonism; IND, indifference; (M) moderate synergism; (S) strong synergism; (SS) super strong synergism. For the FICI model, synergy was defined as a FICI of <0.5, antagonism was defined as a FICI of >4.0, and indifference was defined as a FICI of >0.5 to 4 (i.e., no interaction). For the ∆E model, ∑SYN and ∑ANT were the sums of the percentages of all statistically significant synergistic and antagonistic interactions. Interactions with 100% statistically significant interactions were considered weak synergism, those with 100 to 200% were considered moderate, and those with >200% were considered strong.
FIG. 1. Assessment of the in vitro interaction between aspirin (ASA) and amphotericin B (AMB) against a standard strain of C. parapsilosis (ATCC22019) based on the LA-based models and BI-based models. (AI) Checkerboard showing the percentage of growth for each combination, combinations with more than 50% growth (light grey area), the MIC-2 of aspirin (ASA) and amphotericin B (AMB) (percentages in boldface type) as well as the iso-effective combinations based on which the $\sum FIC$ indices were calculated (dark grey area). (AII) Corresponding isobologram with the additivity line (dashed line). (B) The 3-D (I) and contour (II) plots of the percent synergy calculated with the nonparametric approach, which resulted in 918% synergy. The difference between the predicted and the measured percentage of fungal growth ($\Delta E = E_{predicted} - E_{measured}$) is shown on the $z$ axis.
FIG. 2. Assessment of the in vitro interaction between aspirin (ASA) and amphotericin B (AMB) against a standard strain of C. albicans (YEM30) based on the LA-based models and BI-based models. (A) Checkerboard showing the percentage of growth for each combination, combinations with more than 50% growth (light grey area), the MIC-2 of aspirin (ASA) and amphotericin B (AMB) (percentages in boldface type) as well as the iso-effective combinations based on which the $\sum$FIC indices were calculated (dark grey area), combinations with $\sum$FIC indices lower than 0.5 (underlined percentages) and the combination with the lowest $\sum$FIC-2 index (0.25), corresponding to the FICI-2 (percentage shown in black cell). (AII) Corresponding isobologram with the additivity line (dashed line). (B) The 3-D (I) and contour (II) plots of the percent synergy calculated with the nonparametric approach, which resulted in 927% synergy. The difference between the predicted and the measured percentage of fungal growth ($\Delta E = E_{predicted} - E_{measured}$) is shown on the z axis.
FIG. 3. Assessment of the in vitro interaction between aspirin (ASA) and amphotericin B (AMB) against a clinical strain of C. albicans (CCA10) based on the LA-based models and BI-based models. (A) Checkerboard showing the percentage of growth for each combination, combinations with more than 50% growth (light grey area), the MIC-2 of aspirin(ASA) and amphotericin B (AMB) (percentages in boldface type) as well as the iso-effective combinations based on which the $\sum FIC$ indices were calculated (dark grey area), combinations with $\sum FIC$ indices lower than 0.5 (underlined percentages) and the combination with the lowest $\sum FIC$-2 index (0.25), corresponding to the FICI-2 (percentage shown in black cell). (AII) Corresponding isobologram with the additivity line (dashed line). (B) The 3-D (I) and contour (II) plots of the percent synergy calculated with the nonparametric approach, which resulted in 1225% synergy. The difference between the predicted and the measured percentage of fungal growth ($\Delta E = E_{predicted} - E_{measured}$) is shown on the z axis.
Fig. 4(A). Representative time-kill curves of aspirin (ASA, 2-fold serially diluted) alone and in combination with amphotericin B (AMB, 8 μg/ml (AI), 16 μg/ml (AII), 32 μg/ml (AIII)) against biofilm cells of standard strain of C. albicans (YEM30) versus time.
Fig. 4(B). Representative time-kill curves of aspirin (ASA, 2-fold serially diluted) alone and in combination with amphotericin B (AMB, 8μg/ml) against biofilm cells of clinical strain of C. albicans (CCA10) versus time.

Fig. 4(C). Representative time-kill curves of aspirin (ASA, 2-fold serially diluted) alone and in combination with amphotericin B (AMB, 8μg/ml) against biofilm cells of standard strain of C. parapsilosis (ATCC22019) versus time.
TABLE 1. Susceptibilities of Candida strains against ASA alone and in combination with AMB obtained by the spectrophotometric method.

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*α*MICs of biofilm cells had little change in comparison to planktonic cells for aspirin alone, while the MICs to the corresponding strain increased dramatically for amphotericin B alone. When drugs were used in combination, for planktonic cells of the tested strains, MICs of planktonic cells showed no apparent changes based on the MIC-1, while for biofilm cells of the tested strains, MICs of aspirin and amphotericin B decreased markedly based on the MIC-2. Abbreviations: ASA, aspirin; AMB, amphotericin B; F, planktonic cells; BF, biofilm cells.
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