Interaction of Amiodarone with Azoles Against Aspergillus Planktonic Cells and Biofilms in vitro

Zhimin Duan • Jianbo Tong • Nana Zheng • Rong Zeng • Yuzhen Liu • Min Li

Received: 7 July 2022 / Accepted: 15 September 2022 / Published online: 11 October 2022 © The Author(s), under exclusive licence to Springer Nature B.V. 2022

Abstract Aspergillus spp. is the most common clinical pathogen of invasive fungal infection with high mortality. Existing treatments for Aspergillus spp. infection are still inefficient and accompanied by drug resistance, so it is still urgent to find new treatment approaches. The antiarrhythmic drug amiodarone (AMD) has demonstrated antifungal activity against a range of fungi. This study evaluated the efficacy of AMD in combination with triazoles for Aspergillus spp. infection. We tested the combined effect of AMD and three triazole drugs, namely, itraconazole (ITR), voriconazole (VRC), and posaconazole (POS), on the planktonic cells and biofilms of 20 strains of Aspergillus spp. via a checkerboard microdilution assay derived from 96-well plate-based method. Our results reveal that the combination of AMD with ITR or POS against Aspergillus biofilms has synergistic fungicidal effects. By contrast, the combination of AMD with VRC exhibits no antagonistic and synergistic effects. In this way, the use of AMD in combination with ITR or POS could be an effective adjunctive treatment for Aspergillus spp. infection.

Keywords Aspergillus • Biofilms • Amiodarone • Itraconazole • Voriconazole • Posaconazole

Handling Editor: Mariana Henriques.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11046-022-00672-3.

Zhimin Duan and Jianbo Tong contributed to this article equally.

Y. Liu (✉) Department of Dermatology, the Affiliated Jiangning Hospital of Nanjing Medical University, Nanjing 211100, Jiangsu, China e-mail: liuyuzhen409@126.com

Z. Duan • N. Zheng • R. Zeng (✉) • M. Li (✉) Hospital for Skin Diseases (Institute of Dermatology), Jiangsu Key Laboratory of Molecular Biology for Skin Diseases and STIs, Chinese Academy of Medical Sciences and Peking Union Medical College, Nanjing 210042, Jiangsu, China e-mail: zengrong2010@hotmail.com

M. Li e-mail: limin@pumcderm.cams.cn

J. Tong Department of Dermatology, The First Affiliated Hospital of Nanchang University and Institute of Dermatology, Jiangxi Academy of Clinical Medical Sciences, No. 17 Yongwaizheng Street, Nanchang 330001, Jiangxi, China

M. Li Center for Global Health, School of Public Health, Nanjing Medical University, Nanjing 211166, Jiangsu, China
**Introduction**

*Aspergillus* spp. remain the most common invasive fungal infection pathogens, followed by *Candida* spp. Although new triazole drugs are widely used in treating aspergillosis, the mortality rate for aspergillosis remains high, ranging from 40 to 90\% [1–4]. This high mortality rate is closely related to the formation of *Aspergillus* biofilms. Compared with planktonic morphologies, *Aspergillus* biofilms have increased resistance to antimicrobial agents [5, 6] and enhanced pathogenicity against the host [7, 8]. The control of *Aspergillus* spp. infection is mainly based on antifungal therapy, such as amphotericin B, itraconazole (ITR), voriconazole (VRC) and posaconazole (POS), while the optimal antifungal therapy remains uncertain and need to be explored [9].

The antiarrhythmic drug amiodarone (AMD) exhibits a growth inhibition for a broad range of several diverse fungi (*Candida, Aspergillus, Cryptococcus, Saccharomyces*, and *Fusarium*) [10, 11]. Mechanisms by which AMD is involved in anti-fungal infection include the disruption of Ca\(^{2+}\) homeostasis, the activation of the calcineurin pathway, the accumulation of transcription factor Crz1, etc. [12, 13]. The combination of AMD and triazole drugs shows a synergistic inhibitive effect on the planktonic cells of *Candida albicans* [14, 15] and *Aspergillus niger* [10]. Therefore, it is reasonable and promising to study the killing and inhibitory effects of AMD combined with triazole antifungal drugs ITR, VRC or POS on *Aspergillus* biofilms. In this study, we tested the inhibitory effect of AMD combined with ITR, VRC or POS on 20 *Aspergillus* species (11 *A. fumigatus*, 5 *A. flavus*, 3 *A. terreus*, and 1 *A. niger*). Our study revealed that the use of AMD in combination with ITR or POS could be an effective adjunctive treatment for *Aspergillus* spp. infection.

**Materials and Methods**

**Strains**

*Aspergillus* species isolates (11 *Aspergillus fumigatus*, 5 *A. flavus*, 3 *A. terreus*, and 1 *A. niger*) and *Candida parapsilosis* were stored at the Center of Chinese Medical Fungal Collection. *Aspergillus* strains were isolated from Tongji Hospital of Tongji University. Among them, *A. fumigatus* and *A. flavus* were derived from sputum culture of patients, and *A. terreus* and *A. niger* were derived from bronchoalveolar lavage fluid of patients. All strains were maintained at 4 °C on sabouraud dextrose agar (SDA) slopes and grown on an SDA plate at 37 °C for 72 h to ensure purity and viability. *C. parapsilosis* ATCC 22,019 and *A. flavus* ATCC 204304 were used as quality control strains.

**Antifungal Drug Preparations**

ITR (Sigma-Aldrich Co., St. Louis, MO, USA), VRC (Sigma-Aldrich Co., St. Louis, MO, USA), POS (Selleck chemicals, Houston, TX, USA), and AMD (Sigma-Aldrich Co., St. Louis, MO, USA) were obtained as pure powder. Each drug was dissolved in 100% dimethyl sulfoxide at a final concentration of 1280 μg/ml. The ranges of working concentrations of ITR, VRC, POS and AMD were 0.03–16, 0.03–16 μg/ml, 0.03–16 μg/ml, and 0.5–256 μg/ml in drug susceptibility testing for planktonic cells, respectively. The ranges of working concentrations of ITR, VRC, POS and AMD were 0.5–256 μg/ml, 0.5–256 μg/ml, 0.5–256 μg/ml, and 0.5–256 μg/ml in drug susceptibility testing for biofilms, respectively.

The Minimal Inhibitory Concentrations (MICs) Tests

MICs for planktonic cells were tested according to the guidelines of the Clinical and Laboratory Standards Institute document M38/A2 and defined as the lowest drug concentrations that caused 80% growth inhibition compared with that of the drug-free growth control [16]. The prepared planktonic cells were seeded into 96-well plate (100 μL/well). The working concentration ranges for ITR, VRC and POS are shown above. MICs are defined as the lowest concentration that achieves complete inhibition of growth.

As described previously [17], *Aspergillus* biofilm in vitro could be formed in 96-well plate, and the sessile MIC50 (SMIC50) and sessile MIC80 (SMIC80) were determined by the optical density values from the XTT reduction assay [18]. SMIC50 and SMIC80 mean the antifungal concentrations at which a 50 or 80% decrease in absorbance are tested in comparison to the biofilms formed by the same fungal isolate in the absence of antifungal drug.
The interaction of AMD with ITR, VRC or POS against planktonic cells and biofilms of all strains of *Aspergillus* spp. was evaluated with a checkerboard microdilution assay. The working concentration ranges for ITR, VRC, POS and AMD are shown above. 50 μL of ITR, VRC or POS was inoculated horizontally, while another 50 μL of AMD was inoculated vertically. Results were evaluated after 48 h of incubation at 35 °C. Drug combination interactions were classified according to the Fractional Inhibitory Concentration Index (FICI). Based on these results, FICI values were interpreted as: FICI ≤ 0.5, synergy; 0.5 < FICI ≤ 4.0, indifferent; FICI > 4.0, antagonism [19].

**Fig. 1** Effects of AMD on morphology and viability of *Aspergillus* biofilm. Through CLSM observation (a) and XTT test (b), it was found that AMD exhibited an inhibitory effect on biofilms at concentrations above 64 μg/ml.
Table 1  MICs and FICI values with combinations of AMD with ITR, VRC and POS against planktonic Aspergillus spp

| Strain | MIC80 (µg/ml) | AMD | ITR | ADM/ITR | ADM/ITR FICI | POS | ADM/POS | ADM/POS FICI | VRC | ADM/ VRC | ADM/VRC FICI |
|--------|--------------|-----|-----|---------|--------------|-----|---------|--------------|-----|---------|-------------|
| A. fumigatus |
| AF1  ≥ 256 | 2 | 8/0.25 | S | 1 | 8/0.015 | S | 0.125 | 16/0.125 | N |
| AF2  ≥ 256 | 1 | 8/0.25 | S | 0.5 | 8/0.125 | S | 0.125 | 32/0.125 | N |
| AF3  ≥ 256 | 1 | 8/0.25 | S | 0.5 | 4/0.03 | S | 0.125 | 16/0.125 | N |
| AF4  ≥ 256 | 2 | 4/0.5 | S | 0.5 | 8/0.125 | S | 0.125 | 32/0.25 | N |
| AF5  ≥ 256 | 1 | 8/0.125 | S | 0.25 | 4/0.25 | N | 0.5 | 16/0.125 | N |
| AF6  ≥ 256 | 2 | 8/0.25 | S | 1 | 8/0.06 | S | 0.125 | 8/0.25 | N |
| AF7  ≥ 256 | 2 | 4/0.125 | S | 0.5 | 2/0.125 | S | 0.125 | 8/0.25 | N |
| AF8  ≥ 256 | 4 | 4/1 | S | 0.5 | 2/0.125 | S | 0.125 | 8/0.25 | N |
| AF9  ≥ 256 | 2 | 8/0.25 | S | 0.5 | 4/0.3 | S | 0.25 | 8/0.125 | N |
| AF10 ≥ 256 | 1 | 4/0.125 | S | 0.5 | 8/0.06 | S | 0.5 | 32/0.25 | N |
| AF11 ≥ 256 | 4 | 8/0.25 | S | 0.25 | 4/0.125 | S | 0.5 | 16/0.5 | N |
| A. flavus |
| AFL1 ≥ 256 | 1 | 2/0.5 | S | 0.25 | 8/0.03 | S | 0.5 | 8/0.5 | N |
| AFL2 ≥ 256 | 2 | 4/0.5 | S | 0.5 | 8/0.06 | S | 1 | 16/1 | N |
| AFL3 ≥ 256 | 1 | 4/0.5 | N | 0.25 | 8/0.06 | S | 0.3 | 32/0.3 | N |
| AFL4 ≥ 256 | 0.5 | 4/0.125 | S | 0.5 | 8/0.125 | S | 0.5 | 16/0.5 | N |
| AFL5 ≥ 256 | 2 | 4/0.5 | S | 1 | 8/0.125 | S | 1 | 32/1 | N |
| A. terreus |
| AT1 ≥ 256 | 2 | 4/0.25 | S | 0.5 | 8/0.5 | N | 0.5 | 16/0.5 | N |
| AT2 ≥ 256 | 4 | 2/0.25 | S | 1 | 8/0.25 | S | 1 | 8/0.5 | N |
| AT3 ≥ 256 | 2 | 2/0.5 | S | 0.5 | 8/0.125 | S | 0.125 | 16/0.5 | N |
| A. niger |
| AN1 ≥ 256 | 2 | 2/0.5 | S | 1 | 8/0.015 | S | 0.06 | 32/0.06 | N |

S, synergism (FICI ≤ 0.5); N, no interaction (0.5 < FICI ≤ 4.0)

Confocal Laser Scanning Microscope (CLSM)

Analysis of Biofilms

CLSM (Olympus FV1000) was used to record the images of the biofilms. The biofilms were stained with FUN-1 (Molecular Probes, Eugene, Oregon), which can bind the intravacuolar structures of the fungal cells, according to the manufacturer’s instructions. FUN-1 solution was added at a concentration of 25 µmol/L (200 µL) to the surface of biofilms, followed by incubation at 37 °C for 20 min in the dark. Subsequently, the surface of the biofilms was washed with PBS and observed by CLSM. A laser with an excitation wavelength of 488 nm was used, and the biofilms observed at a magnification of × 200.

Conidia concentrations were determined at the early stage of biofilm formation (10 h) and the biofilm thickness was determined at the late stage (24 h). Biofilm thickness was scanned layer by layer from top to bottom at 1 µm intervals by CLSM. 3D photos of biofilms were built up by 3D Olympus Fluoview software.

XTT Reduction Assay

The viability of the A. fumigatus was evaluated by XTT reduction assay. The XTT/menadione reagent (Sigma-Aldrich, USA) was freshly prepared for each experiment. The XTT/menadione solution was prepared by dissolving 2 mg XTT in 10 mL of PBS, and
then supplemented it with 100 µL of a 10 mM menadione stock solution (0.4 mM in acetone). After incubating the A. fumigatus conidia suspension or biofilms for test, each well of the 96 multi-well plate was filled with 100 µL XTT/menadione solution and incubated at 37 °C for 3 h in the dark. Following incubation, the absorbance was measured at a wavelength of 490 nm using a microplate reader.

Statistics

All assays were performed on at least three independent occasions. GraphPad Prism 7 was used for statistical analyses and graphs. Significance was defined as \( P < 0.05 \).

Table 2  SMICs and FICI values with combinations of AMD with ITR against Aspergillus biofilms

| Strain | SMIC50 (µg/ml) | SMIC80 (µg/ml) |
|--------|----------------|----------------|
|        | AMD | ITR | ADM/ITR | FICI | AMD | ITR | ADM/ITR | FICI |
| A. fumigatus |     |     |        |      |     |     |        |      |
| AF1    | ≥ 256 | 128 | 32/32 | S    | ≥ 256 | 128 | 64/64 | S    |
| AF2    | ≥ 256 | 32  | 64/16 | N    | ≥ 256 | 128 | 64/32 | S    |
| AF3    | ≥ 256 | 64  | 128/32| N    | ≥ 256 | ≥ 256| 128/64| S    |
| AF4    | ≥ 256 | 128 | 32/32 | S    | ≥ 256 | ≥ 256| 32/64 | S    |
| AF5    | ≥ 256 | 32  | 64/16 | N    | ≥ 256 | 128 | 64/32 | S    |
| AF6    | ≥ 256 | 16  | 32/16 | N    | ≥ 256 | 64  | 32/64 | N    |
| AF7    | ≥ 256 | 32  | 64/8  | S    | ≥ 256 | 128 | 64/32 | S    |
| AF8    | ≥ 256 | 128 | 64/32 | S    | ≥ 256 | ≥ 256| 128/64| S    |
| AF9    | ≥ 256 | 64  | 128/64| N    | ≥ 256 | 128 | 64/32 | S    |
| AF10   | ≥ 256 | 32  | 64/16 | N    | ≥ 256 | 64  | 64/16 | S    |
| AF11   | ≥ 256 | 128 | 64/32 | S    | ≥ 256 | ≥ 256| 128/64| S    |

A. flavus

| AFL1   | ≥ 256 | 128 | 64/32 | S    | ≥ 256 | ≥ 256| 64/64 | S    |
| AFL2   | ≥ 256 | 128 | 128/64| S    | ≥ 256 | ≥ 256| 128/32| S    |
| AFL3   | ≥ 256 | ≥ 256| 64/64 | S    | ≥ 256 | ≥ 256| 64/64 | S    |
| AFL4   | ≥ 256 | 32  | 64/32 | N    | ≥ 256 | 64  | 64/16 | S    |
| AFL5   | ≥ 256 | 64  | 64/16 | S    | ≥ 256 | ≥ 256| 64/16 | S    |

A. terreus

| AT1    | ≥ 256 | ≥ 256| 128/128| N    | ≥ 256 | ≥ 256| 128/36| S    |
| AT2    | ≥ 256 | ≥ 256| 64/128 | S    | ≥ 256 | ≥ 256| 128/64| S    |
| AT3    | ≥ 256 | 128  | 64/32 | S    | ≥ 256 | 128 | 64/32 | S    |

A. niger

| AN     | ≥ 256 | ≥ 256| 64/64 | S    | ≥ 256 | ≥ 256| 32/32 | S    |

\( S \) synergism (FICI \( \leq 0.5 \)); \( N \) no interaction (0.5 < FICI \( \leq 4.0 \))

Results

Effects of AMD on Morphology and Viability of Aspergillus Biofilm

CLSM was used to record the entire process of biofilm formation, and the vitality gradually increased with incubation time, as shown in Supplement Fig. 1. A. fumigatus biofilm can be effectively formed in vitro. Through XTT test and CLSM observation, it was found that AMD exhibited an inhibitory effect on biofilms at concentrations above 64 µg/ml, and showed an enhanced inhibitory effect with the increase of AMD concentration in a concentration-dependent manner (Fig. 1).
The ranges of the MICs of ITR, VRC, POS and AMD for the planktonic cells of *Aspergillus* spp. were 0.5–4 µg/ml for ITR, 0.03–1 µg/ml for VRC, 0.25–1 µg/ml for POS, and ≥ 256 µg/ml for AMD (Table 1).

The ranges of the SMIC50 and SMIC80 of ITR, VRC, POS and AMD for *Aspergillus* biofilms were 16 to ≥ 256 µg/ml and 64 to ≥ 256 µg/ml, respectively (Tables 2, 3 and 4). As described in previous studies [17], the susceptibility of biofilms was higher than that of planktonic cells against the three antifungal drugs (ITR, VRC and POS).

### Table 3  SMICs and FICI values with combinations of AMD with POS against *Aspergillus* biofilms

| Strain      | SMIC50 (µg/ml) | SMIC80 (µg/ml) |
|-------------|----------------|----------------|
|             | AMD  | POS | ADM/POS FICI | AMD  | POS | ADM/POS FICI |
| *A. fumigatus* |
| AF1         | ≥ 256 | 32  | 16/4 S       | ≥ 256 | 64  | 64/16 S       |
| AF2         | ≥ 256 | 16  | 8/2  S       | ≥ 256 | 64  | 32/8  S       |
| AF3         | ≥ 256 | 16  | 32/4 S       | ≥ 256 | 32  | 32/8  S       |
| AF4         | ≥ 256 | 32  | 32/4 S       | ≥ 256 | 64  | 8/16 S        |
| AF5         | ≥ 256 | 8   | 16/1 S       | ≥ 256 | 32  | 16/4 S        |
| AF6         | ≥ 256 | 32  | 64/16 N      | ≥ 256 | 128 | 16/32 S       |
| AF7         | ≥ 256 | 8   | 32/2 S       | ≥ 256 | 64  | 32/16 S       |
| AF8         | ≥ 256 | 4   | 64/1 S       | ≥ 256 | 32  | 64/8  S       |
| AF9         | ≥ 256 | 8   | 32/2 S       | ≥ 256 | 32  | 16/4  S       |
| AF10        | ≥ 256 | 8   | 64/2 S       | ≥ 256 | 64  | 32/8  S       |
| AF11        | ≥ 256 | 16  | 32/4 S       | ≥ 256 | 64  | 64/8  S       |
| *A. flavus* |
| AFL1        | ≥ 256 | 4   | 16/1 S       | ≥ 256 | 32  | 64/2  S       |
| AFL2        | ≥ 256 | 8   | 32/2 S       | ≥ 256 | 64  | 32/8  S       |
| AFL3        | ≥ 256 | 8   | 64/2 S       | ≥ 256 | 64  | 16/16 S       |
| AFL4        | ≥ 256 | 32  | 32/8 S       | ≥ 256 | 128 | 64/16 S       |
| AFL5        | ≥ 256 | 128 | 64/64 N      | ≥ 256 | 256 | 64/64 S       |
| *A. terreus* |
| AT1         | ≥ 256 | 128 | 32/64 S      | ≥ 256 | 256 | 32/64 S       |
| AT2         | ≥ 256 | 64  | 64/32 S      | ≥ 256 | 256 | 16/32 S       |
| AT3         | ≥ 256 | 128 | 64/32 S      | ≥ 256 | 256 | 32/64 S       |
| *A. niger*  |
| AN          | ≥ 256 | 64  | 32/8 S       | ≥ 256 | 256 | 32/32 S       |

S, synergism (FICI ≤ 0.5); N, no interaction (0.5 < FICI ≤ 4.0)

Mics and FICI Values With Combinations Of AMD With ITR, VRC, and POS Against Aspergillus spp. Planktonic Cells and Biofilm

The ranges of the MICs of ITR, VRC, POS and AMD for the planktonic cells of *Aspergillus* spp. were 0.5–4 µg/ml for ITR, 0.03–1 µg/ml for VRC, 0.25–1 µg/ml for POS, and ≥ 256 µg/ml for AMD (Table 1).

The ranges of the SMIC50 and SMIC80 of ITR, VRC, POS and AMD for *Aspergillus* biofilms were 16 to ≥ 256 µg/ml and 64 to ≥ 256 µg/ml, respectively (Tables 2, 3 and 4). As described in previous studies [17], the susceptibility of biofilms was higher than that of planktonic cells against the three antifungal drugs (ITR, VRC and POS).

Effects of AMD with ITR, VRC and POS on Morphology and Viability of Aspergillus Biofilm

The biofilms were photographed at 10 and 24 h (Fig. 2a). XTT tests revealed variations in viability during biofilm development (Fig. 2b). ITR, POS and VRC all had inhibitory effects on the adhesion (10 h) and formation (24 h) of biofilms. Combined with AMD, ITR and POS exhibited synergistically enhanced anti-biofilm formation effects. However, the antibacterial effect of VRC in combination with AMD did not change.
Courchesne [11] found that AMD has a potent fungicidal activity against *A. fumigatus* by growth rate test and colony-forming assay. By contrast, the result did not show fungicidal activity alone in our antifungal susceptibility test on planktonic cells or biofilms of *Aspergillus* spp. Guo [14] found that the MIC range of AMD was 512 μg/ml in the in vitro susceptibility of *C. albicans*. The different detection methods may result to different results. However, we observed that the combination of AMD with ITR, VRC or POS against planktonic cells and biofilms of *Aspergillus* spp. had some encouraging results (Tables 1, 2, 3 and 4). In the interaction of AMD with ITR, VRC or POS against planktonic cells of *Aspergillus*, the combination of AMD with ITR displayed a synergistic inhibitory effect in 11 of 11 *A. fumigatus* strains, with FICI values ranging from 0.14 to 0.28, and 7 of 9 non-*fumigatus* *Aspergillus*, with FICI values ranging from 0.13 to 0.26 (Table 1). The synergistic inhibitory effect could also be found in the combination of AMD with POS in 10 of 11 *A. fumigatus* strains, with FICI values ranging from 0.09 to 0.32, and 8 of 9 non-*fumigatus* *Aspergillus*, with FICI values ranging from 0.04 to 0.28 (Table 1). When AMD was combined with VCR, no interaction effect against *Aspergillus* spp. was observed with FICI values ranging from 0.56 to 1.12 (Table 1).

### Table 4 SMICs and FICI values with combinations of AMD with VRC against *Aspergillus* biofilms

| Strain     | SMIC50 (μg/ml) | SMIC80 (μg/ml) |
|------------|----------------|----------------|
|            | AMD  | VRC  | ADM/VRC | FICI | AMD  | VRC  | ADM/VRC | FICI |
| *A. fumigatus* |      |      |         |      |      |      |         |      |
| AF1        | ≥ 256 | 16   | 64/8    | N    | ≥ 256 | 64   | 128/16  | N    |
| AF2        | ≥ 256 | 8    | 32/8    | N    | ≥ 256 | 32   | 64/16   | N    |
| AF3        | ≥ 256 | 8    | 32/8    | N    | ≥ 256 | 64   | 128/32  | N    |
| AF4        | ≥ 256 | 64   | 64/32   | N    | ≥ 256 | 128  | 128/32  | N    |
| AF5        | ≥ 256 | 16   | 32/16   | N    | ≥ 256 | 64   | 128/32  | N    |
| AF6        | ≥ 256 | 32   | 16/32   | N    | ≥ 256 | 64   | 128/16  | N    |
| AF7        | ≥ 256 | 64   | 64/32   | N    | ≥ 256 | 128  | 128/64  | N    |
| AF8        | ≥ 256 | 128  | 64/128  | N    | ≥ 256 | ≥ 256 | 128/128 | N    |
| AF9        | ≥ 256 | 16   | 32/16   | N    | ≥ 256 | 64   | 64/32   | N    |
| AF10       | ≥ 256 | 32   | 32/16   | N    | ≥ 256 | 64   | 32/32   | N    |
| AF11       | ≥ 256 | 64   | 64/32   | N    | ≥ 256 | ≥ 256 | 64/128  | N    |
| *A. flavus* |      |      |         |      |      |      |         |      |
| AFL1       | ≥ 256 | 32   | 8/32    | N    | ≥ 256 | 128  | 128/64  | N    |
| AFL2       | ≥ 256 | 128  | 8/64    | N    | ≥ 256 | ≥ 256 | 128/64  | N    |
| AFL3       | ≥ 256 | 64   | 16/32   | N    | ≥ 256 | 128  | 128/128 | N    |
| AFL4       | ≥ 256 | 8    | 16/4    | N    | ≥ 256 | 64   | 64/64   | N    |
| AFL5       | ≥ 256 | 64   | 32/32   | N    | ≥ 256 | 128  | 128/64  | N    |
| *A. terreus* |      |      |         |      |      |      |         |      |
| AT1        | ≥ 256 | 64   | 32/32   | N    | ≥ 256 | ≥ 256 | 64/128  | N    |
| AT2        | ≥ 256 | 128  | 16/64   | N    | ≥ 256 | ≥ 256 | 128/128 | N    |
| AT3        | ≥ 256 | 64   | 32/64   | N    | ≥ 256 | 128  | 64/64   | N    |
| *A. niger* |      |      |         |      |      |      |         |      |
| AN         | ≥ 256 | 128  | 32/64   | N    | ≥ 256 | ≥ 256 | 128/64  | N    |

*S* synergism (FICI ≤ 0.5); *N* no interaction (0.5 < FICI ≤ 4.0)
In the interaction of AMD with ITR, VRC or POS against biofilms of \textit{Aspergillus}, some attractive results were identified. When ITR was combined with AMD, the SMIC50 ranges of ITR and AMD decreased to 8–64 µg/ml and 32–128 µg/ml, respectively, the SMIC80 ranges decreased to 16–32 µg/ml and 32–128 µg/ml, respectively (Table 2). Based on SMIC50, 5 of 11 \textit{A. fumigatus} strains, with FICI values ranging from 0.375 to 0.5, and 5 of 9 non-\textit{fumigatus Aspergillus}, with FICI values ranging from 0.375 to 0.5, displayed a synergistic effect. Based on SMIC80, 8 of 11 \textit{A. fumigatus} strains, with FICI values ranging from 0.25 to 0.5, and 6 of 9 non-\textit{fumigatus Aspergillus}, with FICI values ranging from 0.25 to 0.38, indicated a synergistic effect. When POS was combined with AMD, the SMIC50 ranges of POS and AMD decreased to 1–64 µg/ml and 8–64 µg/ml, respectively, the SMIC80 ranges decreased to 2–64 µg/ml and 8–64 µg/ml, respectively (Table 3). Based on SMIC50, all strains of \textit{Aspergillus} spp., with FICI values ranging from 0.16 to 0.5, exhibited a synergistic effect. Based on SMIC80, all strains of \textit{A. fumigatus}, with FICI values ranging from 0.28 to 0.5, and 7 of 9 non-\textit{fumigatus Aspergillus}, with FICI values ranging from 0.25 to 0.38, indicated a synergistic effect. When AMD was combined with VCR, no interaction effect against biofilms of \textit{Aspergillus} spp. was determined, with FICI values ranging from 0.51 to 1.5 based on SMIC50 and FICI values ranging from 0.75 to 1.5 based on SMIC80 (Table 4). No antagonism was observed against \textit{Aspergillus} planktonic cells or biofilms with these combinations.

We are the first to study the in vitro interaction effect of AMD with triazole drugs against \textit{Aspergillus} biofilm. Our results revealed that AMD alone did not significantly inhibit the planktonic cells and biofilms of \textit{Aspergillus} spp. However, when AMD was combined with triazole drugs (such as ITR or POS), most of the isolates exhibited a synergistic effect. This mechanism is not clear enough. AMD has been found
to have a potent fungicidal activity against a broad range of fungi by targets calcium and pH homeostasis, which lead to slow growth and poor sporulation on fungi [10, 15]. Triazoles are fungistatic by inhibiting the production of ergosterol, thereby destroying the integrity of the fungal membrane. Gamarra found that AMD combined with fluconazole can significantly dampen the compensatory response pathways for the production of ergosterol [15]. Therefore, we speculate that the synergy of these two drugs may be the mechanism that the break-up of intracellular calcium and pH homeostasis can lead to downregulation of the synthase of ergosterol, whereas the destruction of the cell membrane further results in the destruction of calcium and pH homeostasis.

Overall, our results supported the role for AMD combined with triazole antifungal agents as a novel combination therapy for Aspergillus biofilm-associated fungal infections. More animal test and clinical experience are needed to verify this effect.

Acknowledgements This work was supported by National Nature Science Foundation of China (82173432, 82103749), Nature Science Foundation of Jiangsu province of China (BK20190144), Nanjing Medical University Science and Technology Development Fund Project (NMUB2020154), Huimin Development Plan Project in Jiangning District of Nanjing (2021021NINQKJHMJHXM0133), Jiangsu Provincial “Double Innovation Doctors” Program (JSSCBS20211610) and the Nanjing Incubation Program for National Clinical Research Center (2019060001).

Author Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Zhimin Duan, Jianbo Tong and Nana Zheng. The first draft of the manuscript was written by Zhimin Duan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This work was supported by National Nature Science Foundation of China (82173432, 82103749), Nature Science Foundation of Jiangsu province of China (BK20190144), Nanjing Medical University Science and Technology Development Fund Project (NMUB2020154), Huimin Development Plan Project in Jiangning District of Nanjing (2021021NINQKJHMJHXM0133), Jiangsu Provincial “Double Innovation Doctors” Program (JSSCBS20211610) and the Nanjing Incubation Program for National Clinical Research Center (2019060001).

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study does not involve ethical approval.

References

1. Neofytos D, Horn D, Anaissie E, Steinbach W, Olyaei A, Fishman J, et al. Epidemiology and outcome of invasive fungal infection in adult hematopoietic stem cell transplant recipients: analysis of Multicenter Prospective Antifungal Therapy (PATH) Alliance registry. Clin Infect Dis. 2009;48(3):265–73. https://doi.org/10.1086/595846.
2. Lopez-Medrano F, Fernandez-Ruiz M, Silva JT, Carver PL, van Delden C, Merino E, et al. Clinical presentation and determinants of mortality of invasive pulmonary aspergillosis in kidney transplant recipients: a multinational cohort study. Am J Transplant. 2016;16(11):3220–34. https://doi.org/10.1111/ajt.13837.
3. van Paassen J, Russcher A, In ’t Veld-van Wingerden AW, Verweij PE, Kuijper EJ. Emerging aspergillosis by azole-resistant Aspergillus fumigatus at an intensive care unit in the Netherlands, 2010 to 2013. Euro Surveill. 2016;21(30). https://doi.org/10.2807/1560-7917.ES.2016.21.30.30300.
4. Brakhage AA. Systemic fungal infections caused by Aspergillus species: epidemiology, infection process and virulence determinants. Curr Drug Targets. 2005;6(8):875–86. https://doi.org/10.2174/138945005774912717.
5. Gao L, Sun Y. In vitro interactions of antifungal agents and tacrolimus against Aspergillus biofilms. Antimicrob Agents Chemother. 2015;59(11):7097–9. https://doi.org/10.1128/AAC.01510-15.
6. Seidler MJ, Salvenmoser S, Muller FM. Aspergillus fumigatus forms biofilms with reduced antifungal drug susceptibility on bronchial epithelial cells. Antimicrob Agents Chemother. 2008;52(11):4130–6. https://doi.org/10.1128/AAC.00234-08.
7. Suh JD, Ramakrishnan V, Palmer JN. Biofilms. Otolaryngol Clin North Am. 2010;43(3):521–30, viii. https://doi.org/10.1016/j.cot.2010.02.010.
8. Johnson CJ, Cabezas-Olcoz J, Kernien JF, Wang SX, Beebe DJ, Huttenlocher A, et al. The Extracellular Matrix of Candida albicans biofilms impairs formation of neutrophil extracellular traps. PLoS Pathog. 2016;12(9): e1005884. https://doi.org/10.1371/journal.ppat.1005884.
9. Glampedakis E, Cassaing F, Bekkar A, Dannaoui E, Bougnoux ME, Bretagne S, et al. Invasive Aspergillosis Due to Aspergillus Section Usti: a multicenter retrospective study. Clin Infect Dis. 2021;72(8):1379–85. https://doi.org/10.1093/cid/ciaa230.
10. Bagar T, Bencina M. Antiarrhythmic drug amiodarone displays antifungal activity, induces irregular calcium response and intracellular acidification of Aspergillus niger - amiodarone targets calcium and pH homeostasis of A. niger. Fungal Genet Biol. 2012;49(10):779–91. https://doi.org/10.1016/j.fgb.2012.07.007.
11. Courchesne WE. Characterization of a novel, broad-based fungicidal activity for the antiarrhythmic drug amiodarone.
12. Gupta SS, Ton VK, Beaudry V, Rulli S, Cunningham K, Rao R. Antifungal activity of amiodarone is mediated by disruption of calcium homeostasis. J Biol Chem. 2003;278(31):28831–9. https://doi.org/10.1074/jbc.M303300200.

13. Oliveira NK, Frank LA, Squizani ED, Reuwsaat JCV, Marques BM, Motta H, et al. New nanotechnological formulation based on amiodarone-loaded lipid core nanocapsules displays anticytotoxic effect. Eur J Pharm Sci. 2021;162: 105816. https://doi.org/10.1016/j.ejps.2021.105816.

14. Guo Q, Sun S, Yu J, Li Y, Cao L. Synergistic activity of azoles with amiodarone against clinically resistant Candida albicans tested by checkerboard and time-kill methods. J Med Microbiol. 2008;57(Pt 4):457–62. https://doi.org/10.1099/jmm.0.47651-0.

15. Gamarra S, Rocha EM, Zhang YQ, Park S, Rao R, Perlin DS. Mechanism of the synergistic effect of amiodarone and fluconazole in Candida albicans. Antimicrob Agents Chemother. 2010;54(5):1753–61. https://doi.org/10.1128/AAC.01728-09.

16. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved Standard CLSI M38-A2. 2nd ed. Clinical and Laboratory Standards Institute; 2008.

17. Zeng R, Li M, Chen Q, Wang L, Zhan P, Wang C, et al. In vitro analyses of mild heat stress in combination with antifungal agents against Aspergillus fumigatus biofilm. Antimicrob Agents Chemother. 2014;58(3):1443–50. https://doi.org/10.1128/AAC.01007-13.

18. Pierce CG, Uppuluri P, Tristan AR, Wormley FL Jr, Mowat E, Ramage G, et al. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat Protoc. 2008;3(9):1494–500. https://doi.org/10.1038/nprot.2008.141.

19. Odds FC. Synergy, antagonism, and what the checkerboard puts between them. J Antimicrob Chemother. 2003;52(1):1. https://doi.org/10.1093/jac/dkg301.

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.