Research in Microbiology 172 (2021) 103873

Contents lists available at ScienceDirect

Research in Microbiology

journal homepage: www.elsevier.com/locate/resmic

Original Article

Improved eradication efficacy of a combination of newly identified antimicrobial agents in *C. albicans* and *S. aureus* mixed-species biofilm

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ARTICLE INFO

Article history:
Received 10 March 2021
Accepted 27 July 2021
Available online 10 August 2021

Keywords:
Polymicrobial biofilms
Chalcone-based derivative
Polycyclic anthracene-maleimide adduct
Antimicrobial combinations
Synergy

SUMMARY

Candida albicans and *Staphylococcus aureus* are common human pathogens, frequently isolated independently or co-isolated from bloodstream infections, and able to form dense polymicrobial biofilms on various medical devices resulting in strong resistance to conventionally used antimicrobials. New and innovative approaches are therefore needed to ensure the successful management of biofilm related infections. In this study, a chalcone-based derivative and a polycyclic anthracene-maleimide adduct, previously ascertained by us as inhibitors of *C. albicans* and *S. aureus* growths, respectively, were reconsidered in a new perspective by evaluating the efficacy of a combined treatment against a polymicrobial biofilm. Both quantitative and qualitative analyses were carried out to delve into their inhibitory potential on the polymicrobial population. Our results indicate that these newly identified antimicrobials are effective in reducing the biomass of the mixed *C. albicans-S. aureus* biofilm and the viability of fungal-bacterial cells within the polymicrobial community; in addition, confocal laser scanning microscopy demonstrates that the combined treatment thoroughly modifies the architecture of the dual-species biofilm.

1. Introduction

Biofilms are well-structured population of microbial cells that are enclosed in a self-produced extracellular polymer matrix, adherent to virtually all biotic or abiotic surfaces. Today there is absolutely no doubt that microbial cells within the biofilm have an increased antibiotic tolerance and virulence. The clinical scenario suggests that biofilms occur *in vivo* with almost more than 75% of human infections recognized to be biofilm-related [1–3]. Although some infections are caused by a single species pathogen, polymicrobial biofilms of fungal and bacterial cells have been identified frequently [4]. This event often renders both the identification of the etiological agents and the treatment of the infection more difficult.

The interspecies interactions among phylogenetically diverse microbes have variable impact on polymicrobial compositions, ranging from synergistic relationships to growth competition during the biofilm development process [5–7]. In this context, microorganisms could exhibit altered sensitivity to antimicrobial agents [8], leading to a worsen disease progression [9].

Among the fungal species, *Candida albicans* is the most common human pathogen causing diseases ranging from superficial mucosal to life-threatening systemic infections mainly associated with its ability to form biofilm. In some niches in the host, *C. albicans* coexists with different bacterial species, and it is able to form polymicrobial biofilms with many partners including *Staphylococcus aureus* [5,10]. Although *S. aureus* is a poor former of biofilms compared to other staphylococci, it substantially increases its biofilm capability in the presence of *C. albicans*, as the fungus creates a scaffold for the bacteria [11]. *Candida* spp. and *S. aureus* mixed infections may occur in several body districts, including skin, lung,

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https://doi.org/10.1016/j.resmic.2021.103873
0923-2508/

oral cavity, as well as blood stream as a consequence of the colonization of medical devices [12–15].

The chemotherapeutic treatment of the polymicrobial biofilms requires a combination of antifungal agents and antibiotics, but it has been reported that such a combination strategy shows poor efficacy and most of which suffer from several unwanted effects [9,10]. In this scenario, the development of novel therapeutic options represents a challenging issue. Valuable in this respect could be the so-called "privileged structures", a number of versatile scaffolds able to interact with different biological targets. Their appropriate functionalization or inclusion into more complex structures provides a viable way to improve the biological potential and develop new bioactive compounds [16]. Recently, we reported newly designed derivatives endowed with peculiar and selective activity against *Candida* spp. and *S. aureus* [17,18]. In particular, the new compounds are characterized by privileged structures-related scaffolds namely a chalcone core and a polycycle adduct between anthracene and maleimide. The biological impact of naturally occurring chalcones, largely widespread in the plant kingdom, is universally known, as they proved to be endowed with a wide spectrum of biological effects, including antioxidant, antibacterial, antiprotozoal, antimutagenic, antimitotic, antimetastatic, and anti-inflammatory [19]. This relevant therapeutic potential is connected to the modulation of a wide range of biomolecular functions. Extensive studies have been performed to elucidate the mechanism of action of this class of compounds, which is principally connected to the *trans*-α,β-unsaturated carbonyl structural motif. This Michael acceptor system can likely act as a nucleophile scavenger establishing covalent linkages with the cysteine residues of several target proteins. This scaffold has been extensively exploited in medicinal chemistry to design and synthesize libraries of compounds characterized by an improved biological profile.

Besides, our research group has recently reconsidered the versatile and easily affordable abovementioned polycycle adduct obtained by applying the one-step Diels–Alder cycloaddition. Indeed, this structure can easily be modified by a suitable decoration of the anthracene derivative and/or by the alkylation of the amide nitrogen with properly selected side chains. This ability has been exploited in order to improve the activity and evaluate the structure–activity relationships, starting from a prototype selected from an in-house library of compounds and showing a promising antibacterial activity. The suggested mechanism could be related to the ability to affect the integrity of the bacterial membrane, probably by means of the inhibition of enzymes involved in membrane redox homeostasis. Notably, several studies demonstrate the versatility of this promising scaffold in various therapeutic areas [20].

Given these observations, one of the previously discovered chalcone-based derivatives (compound **1,** Fig. 1) and a polycyclic anthracene-maleimide adduct (compound **2,** Fig. 1) were reconsidered in a new perspective by evaluating *in vitro* the efficacy of a combined treatment against a mixed biofilm of *C. albicans* and *S. aureus*, and new experiments were herein designed. Compound **1** is an antifungal agent with remarkable anti-virulence activity; it is able to hamper the production of hyphae and biofilm thus it interferes with *C. albicans* pathogenesis [17]. The presence of the small and highly electronegative fluorine atom in the structure provides a peculiar chemical behaviour to the molecule, by granting some favourable properties, among which enhanced metabolic stability, selectivity, and efficacy in target binding. Compound **2** is a potent inhibitor of *S. aureus,* acting as a membrane-disrupting agent, probably through the inhibition of enzymes involved in preserving membrane redox homeostasis and energy production [18].

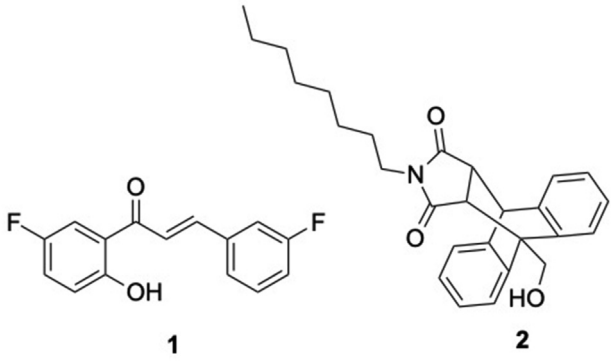

Fig. 1. Chemical structures of the chalcone-based derivative (compound **1**) and the polycyclic anthracene-maleimide adduct (compound **2**), previously discovered by us [17,18].

The present study was aimed to delve into the anti-biofilm properties of the two newly identified agents in a mixed *C. albicans-S. aureus* biofilm; both quantitative and qualitative analysis was carried out to measure the inhibitory potential of compounds **1** and **2** combined treatment on the polymicrobial population, to define the individual activity of each agent on the overall reduction of the biofilm matrix and to investigate the impact of the treatment on biofilm architecture.

2. Materials and methods

2.1. Strains and growth conditions

In vitro experiments were carried out with the *C. albicans* strain ATCC 10231 (American Type Culture Collection) and with the clinical isolate MSSA 1 of *S. aureus*, previously demonstrated as a slime producer [18]. *C. albicans* and MSSA 1 were routinely cultured in Sabouraud dextrose agar and in 5% blood agar, respectively, at 37 °C; Brain Heart Infusion broth (BHI, Sigma–Aldrich) was used for liquid overnight growths while supplemented with 10% Fetal Bovine Serum (FBS, Sigma–Aldrich) for culturing biofilms; Mannitol Salt Agar plates (Biolife Italia) and Chromogenic Candida Agar plates (Biolife Italia) were used for selective regrowth of microbial species following the treatment of the polymicrobial biofilms.

2.2. Antimicrobial agents

The selected compounds (Fig. 1) were prepared as reported in previous papers [17,18]. Briefly, the chalcone-based derivative **1** was obtained by reacting the 5′-fluoro-2′-hydroxyacetophenone and 3-fluorobenzaldehyde in EtOH and in the presence of aqueous solution of KOH. On the other hand, compound **2** was prepared applying the Diels–Alder cycloaddition to anthracen methanol and maleimide, followed by alkylation of the amide nitrogen with bromo-octane in the presence of potassium *ter*-butoxide. Both these easily affordable synthetic routes enable performing further structural modifications in order to improve the pharmacological/toxicological profile of the molecules.

For *in vitro* experiments, the dry powder of the compounds were resuspended in dimethylsulfoxide (DMSO) at 20 mM and used as stock solutions. Compounds were used in the range 3.125–200 μM in a preliminary set of experiments while the working concentrations of 53 μM for compound **1** and 4 μM for compound **2** were used in the combined treatment. These concentrations correspond to the

IC_{50} values previously measured for *C. albicans* and *S. aureus* [17,18]. A final concentration of 0.25% DMSO was used in all analyses and it did not interfere neither with microbiological investigations nor with cellular assays.

2.3. Cell viability and cytotoxicity assays

Compounds were assayed for their cytotoxic effects on african green monkey kidney cells (Vero ATCC CCL-81); both cell viability and lactate dehydrogenase enzyme (LDH) release were evaluated on mammalian cells upon 48 h of treatment. Briefly, 24 h before experiments, cells were seeded into 96-well plates at 10^4 cells/well, and grown in regular culture medium (Eagle's Minimal Essential Medium, 10% FBS (Fetal Bovine Serum), 100 U/mL penicillin, and 100 μg/mL streptomycin), at 37 °C and 5% CO_2. Following washes with phosphate-buffered saline (PBS), cell monolayer was incubated with a renewal complete medium supplemented with either derivative **1** at 53 μM or compound **2** at 4 μM, or both. After 48 h of incubation, culture medium was collected from each well, the monolayer was washed with PBS, and 100 μL of fresh medium containing 10 μL of CCK-8 solution were added (CCK-8, Cell Counting Kit-8, Dojindo Molecular Technologies). The OD (Optical Density) at 450/630 nm were measured after 2 h of incubation and data were expressed as percentage values of cell viability relative to the untreated controls. In parallel, the collected cell-free supernatants were assayed for LDH released through damaged plasma membranes by adding a volume of the reconstituted working solution (Cytotoxicity LDH Assay kit-WST, Dojindo Molecular Technologies). After 30 min of incubation at room temperature in the dark, the stop solution was added and OD at 490 nm measured. Data were expressed as percentage values relative to both the 100% lysis controls, included in the test, and untreated controls.

2.4. Planktonic antimicrobial assay

The chalcone-based derivative **1** and the polycyclic anthracene-maleimide compound **2** were assayed on *C. albicans* and *S. aureus* planktonic cells by a standardized 96-well plate method [17,18], with slight modifications. Briefly, each microbial suspension, prepared in PBS at 10^6 CFU/mL, was incubated with serial 2-fold dilutions of the compound **1** or compound **2** (3.125–200 μM), in a final volume of 200 μL/well. Both strains were assayed in BHI medium. Following 24 h at 37 °C, OD values at 630 nm were measured, and IC_{50} values were interpolated on the generated dose–response curves.

2.5. Biofilm antimicrobial assay

The biofilms were developed on polystyrene 24-well plate by layering 300 μL of an overnight culture of *S. aureus* or *C. albicans* at a density of 10^5 CFU/mL and adding the same of BHI medium for mono-species biofilm, while the dual-species biofilm was developed by inoculating 300 μL of each culture to the well, reaching a total volume of 600 μL. Plate was incubated at 37 °C for 90 min to promote bacterial and fungal adhesion, thereafter wells were slowly rinsed with PBS to remove non-attached cells. Then, 600 μL of BHI medium supplemented with FBS containing both antimicrobial agents (**1** at 53 μM and **2** at 4 μM) were added to each well and plate was incubated for 24 h at 37 °C in static condition to induce biofilm formation. The biomass of the biofilms was assessed by a standardized crystal violet (CV) staining. Briefly, wells were extensively washed in PBS to remove planktonic cells, and the plate was incubated for 1 h at 60 °C. CV solution (0.1% in water) was added to each well and incubated for 15 min at room temperature. The unbound CV was removed by repeated washes with water; finally, the bound CV was resolubilized in 95% ethanol; following 30 min of incubation at room temperature, CV solutions were recovered from the different wells and the OD was read at 550 nm. The efficacy of the compounds to inhibit biofilm formation was expressed as percentage values relative to the untreated growth controls.

The anti-biofilm activity of the combined treatment was also evaluated by adding the antimicrobial agents (**1** at 53 μM and **2** at 4 μM) to biofilms after 24 h of development. Briefly, mono- and dual-species biofilms were developed on polystyrene 24-well plate as described above; after the adhesion phase and biofilm growth for 24 h, compounds were added to the wells and the plate was incubated at 37 °C for an additional 24 h. The activity of the agents on the mono- and dual-species biofilms was quantitatively estimated by CV staining as previously described, and results reported as percentage values compared to controls.

2.6. CFU assay

Polymicrobial biofilms were developed on a polystyrene 24-well plate as previously described, in presence of the agents at their IC_{50} values, and without as untreated control. Following 24 h of incubation, biofilms were extensively washed with PBS and treated with 1 mL of 0.1% dithiothreitol (DTT) (Sputasol, Oxoid) for 10 min at 37 °C to dislodge microbial cells from the matrix [21]. Detached cells were then recovered and plated for CFU count. In particular, serial dilutions of the suspensions were made in PBS and plated on Mannitol Salt Agar (for *S. aureus* enumeration) and Chromogenic Candida Agar (for *C. albicans* enumeration). After 24–48 h at 37 °C, plates were imaged and colonies were digitally counted using the VersaDoc Image System and the colony counting software (BioRad). The same experimental setting was applied on a preformed 24-h polymicrobial biofilm.

2.7. Analysis of the interaction

The nature of the interaction between the chalcone-based derivative **1** and the antibacterial agent **2** in a polymicrobial environment was analyzed by a simplified checkerboard synergy testing assay. Briefly, microbial cells were allowed to attach to the polystyrene 24-well plate as previously described, thereafter different concentrations of the compounds were added and after 24 h of incubation, biomasses were evaluated by CV staining. In particular, a 3 × 3 matrix of concentrations (IC_{50} × 2, IC_{50}, no compound) was applied to polymicrobial biofilms. The same methodology was used to treat a preformed 24-h biofilm For each experimental condition, the OD at 550 nm was measured and results were reported as percentage values compared to untreated controls. The interaction of the compounds was defined as synergy when the percentage values obtained on biofilms treated with both compounds resulted higher than the sum of the individual effects of each compound on the mixed biofilm.

2.8. Confocal laser scanning microscopy (CLSM) analysis

For microscopy analysis, polymicrobial biofilms were formed as previously described over a period of 24 h in permanox chamber slides (Thermo Scientific Nunc Chamber Slide System). Biofilms were produced in BHI/FBS 10% containing both antimicrobial agents at their IC_{50} values, and without as untreated control. After 24 h of growth, biofilms were extensively washed in PBS, dried, and fixed on the plastic support by incubation with 100% methanol for 2 min. Then, fluorescent markers were added directly to the chamber slides: SYTO9 (Molecular Probes) a nucleic acid-binding stain labeling both bacterial and fungal cells [22], and Calcofluor

white (Calcofluor White Reagent Dropper, BD), a fluorescent blue dye that binds β-1,3 and β-1,4 polysaccharides in the fungal cell walls. The stained biofilms were washed twice with PBS before imaging.

Confocal fluorescence imaging was performed on a Nikon A1 system with an inverted Ti-E microscope (Nikon Company). The confocal fluorescence microscope Nikon A1 is equipped with a Picoquant CW/pulsed diode laser and an argon ion CW laser, for excitation at 405 and 488 nm, respectively. Images were collected using a Nikon Plan Apo VC 60 × oil immersion objective with NA 1.40. Filters were set to register the fluorescence of SYTO9 or Calcofluor white in the 450/50 nm and 525/50 nm, ranges. DIC images completed the imaging part. Where useful the LUT (Look-up-table) intensity scale is visualized in the confocal fluorescence images.

2.9. Statistical analysis

For biofilm investigations, 3 wells for each treatment regimen were assayed and at least two independent experiments were performed. For cell viability and cytotoxicity assays, 3 wells for each condition were tested and three independent experiments were carried out. Percentage values were obtained as relative to the untreated controls. Differences between samples were determined by unpaired student's t test, or one-way ANOVA followed by Dunnett's Multiple Comparison Test using the GraphPad Prism Software, version 6.0 (GraphPad Software). Statistical significance was set at p values < 0.05, indicated by asterisks in figures.

3. Results and discussion

3.1. Polymicrobial biofilms

In the present study, all experiments aimed to quantitatively evaluate biofilm biomasses were carried out on polystyrene 24-well plates instead of the conventional 96—well plates, harboring microbial species at 10^5 CFU/mL and culturing cells in BHI broth supplemented with 10% FBS for 24—48 h. This experimental model was selected in order to allow cross-kingdom biofilm development without removal of cells during the culture medium exchange, thus to correctly measure their biomass after treatments. In addition, FBS was added to the culture medium to mimic the human physiological environment.

A preliminary set of experiments indicated that co-incubation of *C. albicans* and *S. aureus* allowed for the development of a higher amount of biofilm biomass that the sum of each mono-species biofilm (Supplementary Fig. 1). The synergistic increase of the biomass is possibly related to the culturing medium used herein; indeed, BHI broth supplemented with FBS stimulates yeast-to-hyphae morphogenesis of *C. albicans,* this in turn promotes *S. aureus* biofilm production, as germ tubes and hyphal forms act as scaffold for bacterial adhesion and aggregation. It has been reported that *S. aureus* directly adheres to the Als3 protein of *C. albicans* to form a complex structure with increased mutually beneficial interactions, enhanced antimicrobial tolerance and protection against host defences [4,10,23,24].

In the present study, the physical interaction between *C. albicans* and *S. aureus* in the mixed biofilm was demonstrated by CLSM analysis, where images showed adherence to and clumping of staphylococci around *C. albicans* hyphae, forming thick biofilm aggregates (Fig. 2).

3.2. Activity and selectivity of the compounds

The antimicrobial susceptibility of the newly identified antimicrobial agents was assessed both on planktonic cells and biofilms of *C. albicans* and *S. aureus* in order to validate their inhibitory properties and to ascertain their selectivity in the present experimental setting. The IC_{50} values resulted 53 μM for compound **1** against *C. albicans* and 4 μM for compound **2** against *S. aureus* confirming the previously obtained results, thus the activity of the antimicrobials in the herein used medium (BHI instead of RPMI-1640 medium for *C. albicans* and Mueller Hinton broth for *S. aureus*). Neither the antifungal compound nor the antibacterial agent interfered with the growth of *S. aureus* and *C. albicans,* respectively (Supplementary Fig. 2). In addition, no cross-activity was measured on the monomicrobial biofilms when tested with compounds at their IC_{50} values (Supplementary Fig. 3), thus proving the specific activity of both antimicrobials.

3.3. Cell viability and cytotoxicity

Having assessed the activity and selectivity of the compounds at their IC_{50} values, they were used in mammalian cell studies. The antimicrobial agents were used as single agent at 53 μM for compound **1** and 4 μM for compound **2**, and in a combined treatment for 48 h. Two different assays were carried out to measure both cell viability and cell membrane damage. As single treatment, compounds did not display any toxicity, while the combined treatment exerted a certain degree of cytotoxicity; in particular, at tested concentrations, cell viability was reduced of 39.4% compared to the positive controls, but LDH activity, a marker of cell death, increased to the 22.8% (Fig. 3A,B). Percentage values lower than 25% are acceptable, thus indicating a good safety profile [25].

3.4. Combined treatment of mono-species biofilm

In previous studies, the anti-biofilm activity of each compound was established against a single species biofilm and in different experimental settings, including culturing conditions (RPMI-1640 medium for *C. albicans* and Mueller Hinton broth for *S. aureus*), and methodologies (CV staining and viability assay) [17,18]. Herein, newly investigations were therefore performed as BHI broth, a nutrient-rich media, and FBS were used to produce the biofilms, possibly modifying the efficacy of the antimicrobial agents. In addition, CV assay was selected as method of choice for biofilm assessment, as it measures the amount of the polymeric matrix instead of the metabolic activity of cells. It is known that fungal and bacterial cells account only for 5—35% of biofilm volume, and the remaining is extracellular matrix, thus assays measuring the viability of biofilm cells may not accurately define the properties of compounds in terms of biofilm inhibition and eradication, and lack in reliability at lower numbers of cells [26].

The efficacy of the combined treatment was determined on mono-species biofilms of *C. albicans* and *S. aureus* by adding both agents at their IC_{50} values during biofilm development. Compounds significantly inhibited the biofilm formation compared to the untreated controls with percentage values of 58.5 ± 2.1% for *C. albicans* and 60.2 ± 9.1% for *S. aureus* (Fig. 4A). In addition, they displayed a moderate activity towards the pre-form mono-species biofilms reducing the biomasses by the 34.5 ± 1.8% and 26.7 ± 0.9% for *C. albicans* and *S. aureus*, respectively (Fig. 4B).

3.5. Combined treatment of C.albicans-S.aureus biofilm

Having assessed the activity on the mono-species biofilms, the efficacy of compounds **1** and **2** was evaluated on the mixed *C. albicans-S. aureus* biofilm. Derivative **1** at 53 μM and compound **2** at 4 μM were added to BHI broth supplemented with 10% of FBS and the biomasses of the biofilms were quantitatively measured following 24 h of incubation. The combined use of these

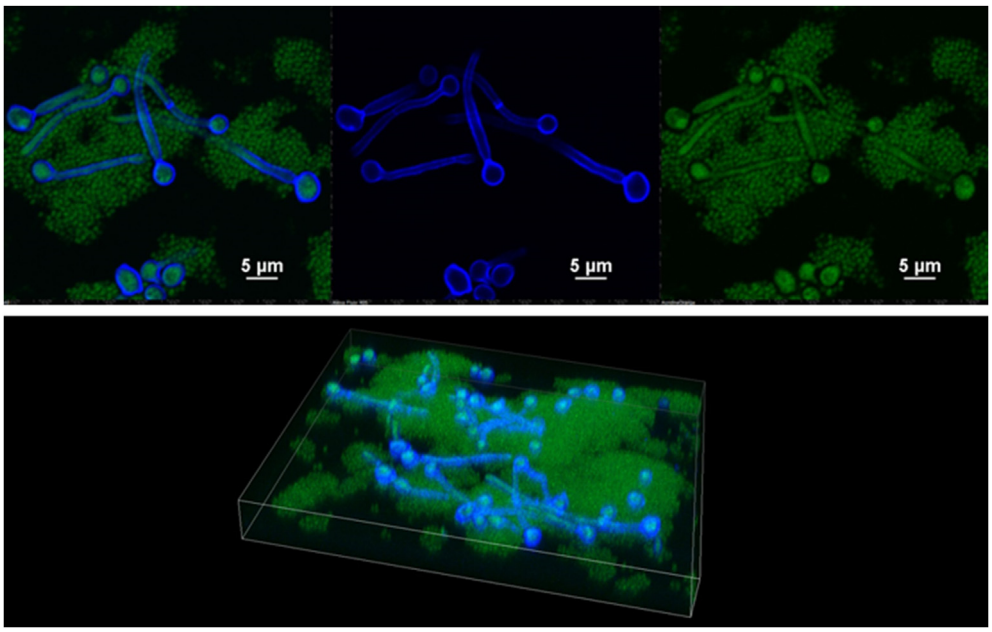

Fig. 2. CLSM images (Top) and 3D reconstruction of Z-stack over 10 µm (Bottom) of mixed biofilm formations. Biofilms were stained with Calcofluor white and SYTO9 collecting fluorescence at 450 nm (blue images) and 525 nm (green) together with a merged image. These results demonstrate that *S. aureus* adheres to and forms biofilm aggregates around *C. albicans* hyphae. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

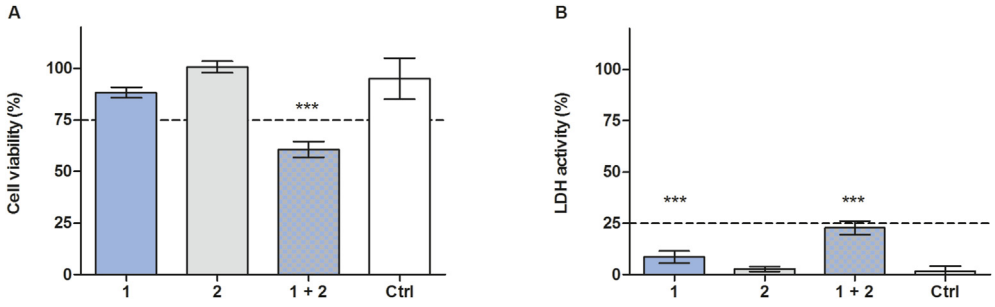

Fig. 3. Vero cells cultured for 48 h in presence of the chalcone-based derivative (**1**), the polycyclic anthracene-maleimide adduct (**2**), and both antimicrobials (**1** + **2**). The combined treatment significantly decreases cell viability (A) whereas LDH activity (B), index of cell death, remained acceptable (less than 25%) suggesting a cytostatic effect on cell proliferation rather than a cytotoxic activity. Percentage values are relative to the untreated cells.

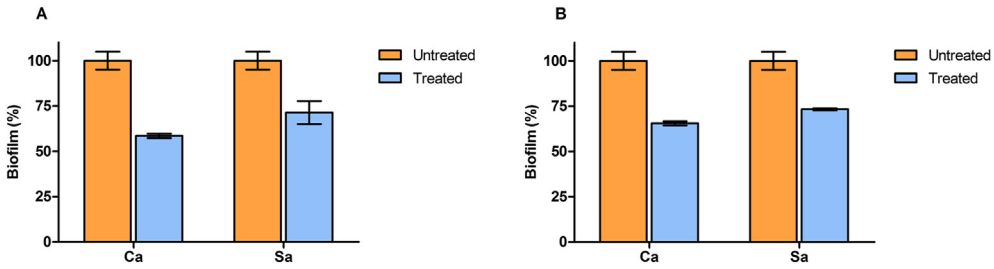

Fig. 4. Anti-biofilm activity of the combined treatment on the mono-species biofilms. Compounds were used at their IC_{50} values and their inhibitory properties were evaluated on biofilm formation (A) and on a preformed 24-h biofilm (B).

compounds significantly inhibited the development of the mixed bacterial-fungal biofilm compared to the untreated control with a percentage value of 62.4 ± 9.3%. The nature of the interaction between the two agents was analyzed by measuring the biomasses of the polymicrobial biofilms treated with different antimicrobial concentrations. A 3 × 3 matrix of concentrations was applied, and the percentage values of biofilm inhibition obtained in each experimental condition, compared to the untreated control, were reported in Fig. 5A. In this set of experiments, the inhibitory activity of the agents at their IC_{50} values proved to be more than 2-fold the sum of the individual effect of the compounds (11.1% and 13.3%). Thus, the chalcone-based derivative **1** and the antibacterial agent **2** displayed a synergistic antimicrobial activity, at a non cytotoxic concentration. The same experimental approach was used to

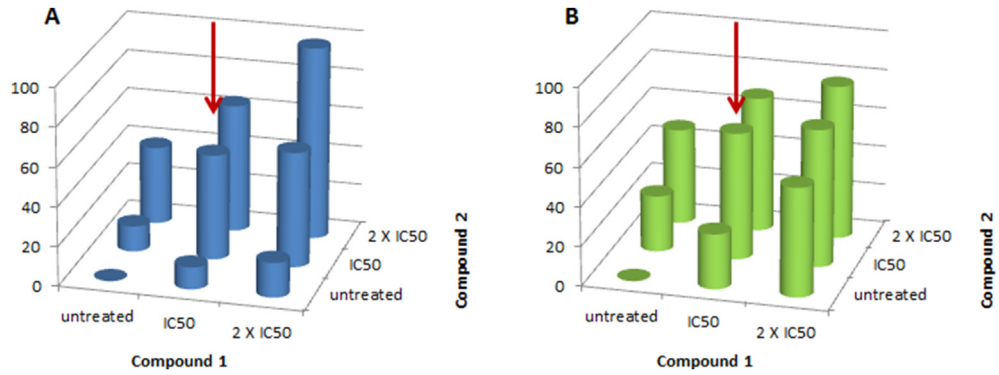

Fig. 5. Percentage values of biofilm inhibition obtained by using different combination of compounds against the polymicrobial biofilm. Red arrows indicate the effect of the combined agents at their IC_{50} values. In both experimental conditions (A: biofilm formation; B: biofilm disruption), the overall inhibition is higher than the sum of the individual effect of the compounds. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

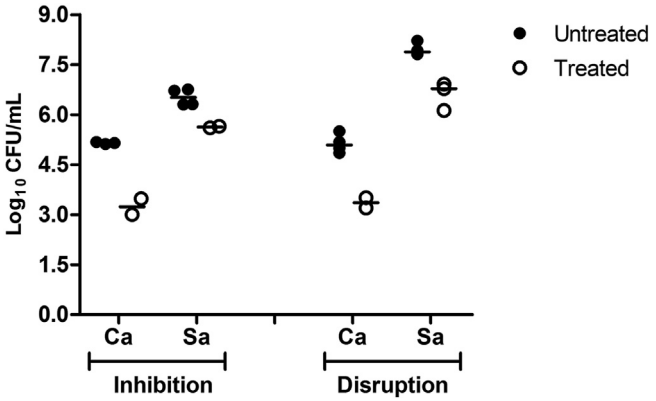

Fig. 6. CFU counts of each microbial species following the combined treatment of the polymicrobial biofilm. Cells were recovered from the biofilms and regrowth on selective agar plates. In both experimental conditions, biofilm inhibition and disruption of a pre-formed biofilm, *C. albicans* displays a higher susceptibility to the killing of the antifungal compound.

evaluate the activities of the combined treatment on pre-form polymicrobial biofilms (Fig. 5B). The overall biomass reduction of 64.4 ± 6.2% was higher than the sum of the single activity (27.9% and 28.5%), however, the synergy between the compounds was less marked compared to their activity on the biofilm development.

3.6. CFU assay

To delve into the anti-biofilm properties of the compounds, CFU counts on selective agar plates were carried out on cells detached from the *C. albicans-S. aureus* biofilm community. Treated and untreated biofilms were disrupted by adding the DTT reducing agent, and viable cells were plated on Mannitol Salt Agar and Chromogenic Candida Agar for regrowth. The combined use of the antifungal compound **1** and antibacterial agent **2** significantly reduced the viability of microbial species compared to untreated controls (Fig. 6). The treatment was more effective in reducing *C. albicans* from the polymicrobial biofilm compared to *S. aureus* reduction. Indeed, both species within the polybiofilm were susceptible to the killing by the agents, but CFU measurements indicated a 1.9 Log-unit reduction for *C. albicans* and 1.1 Log-unit reduction for *S. aureus.* The same experimental setting was applied to evaluate the effectiveness of the compounds after biofilm development. CFU counts allowed measuring a higher killing activity of the combined treatment on *C. albicans* compared to *S. aureus* (1.8 and 1.3 Log-unit reduction for *C. albicans* and *S. aureus,* respectively). In the present experimental conditions, the combined use of the chalcone-based derivative **1** and antibacterial agent **2** mainly affected fungal viability compared to bacterial viability suggesting that *S. aureus* within the biofilm was more resistant to the killing. A possible explanation is that *S. aureus* becoming encased in the biofilm matrix, during the polymicrobial growth, enhanced tolerance to the polycyclic anthracene-maleimide compound **2**. This finding has been previously reported for commonly used antibiotic drugs [27].

3.7. Confocal laser scanning microscopy

Confocal laser scanning fluorescence microscopy was performed on dual-species biofilms treated with the antimicrobials, and untreated as control. Two markers were used to stain the biofilms: SYTO9, allowing the detection of nucleic acids and Calcofluor white, targeting the polysaccharides within the fungal cell wall. As expected Calcofluor white colors the walls of the fungal cells and the extended hyphae of *C. albicans*. SYTO9 colored the nucleic acid material of both species. Noticeably the treatment with agents **1** and **2** caused a strong reduction in fluorescence intensity of both fluorophores (Supplementary Fig. 4). In the case of *C. albicans* the reduced fluorescence intensity of Calcofluor white may indicate the derivative **1** activity in terms of decreased polysaccharide synthesis, or chitin and β-1,3 glucan degradation. Indeed, the proposed mechanism of action of chalcones involves the inhibition of both 1,3 β-glucan synthase and chitin synthase [28]. For both species the reduced fluorescence intensity of SYTO9 is likely indicating reduced amounts of nucleic acids within cells possibly due to inhibition or degradation of DNA-RNA and leaks in the cell membranes. Indeed, we previously demonstrated that the polycyclic anthracene-maleimide compound **2** has membrane-disrupting activity on *S. aureus* cells, leading to nucleic acids loss through the damaged cytoplasmic membrane.

Importantly, CLSM images in Figs. 7 and 8 clearly revealed that the combined treatment led to a considerable reduction of biomass and cell aggregates together with a significant decrease in the amount of hyphae forms of *C. albicans.* The number of *C. albicans* cells was not significantly decreasing but yeast−hyphal transition was remarkably impaired in line with the proposed activity of the

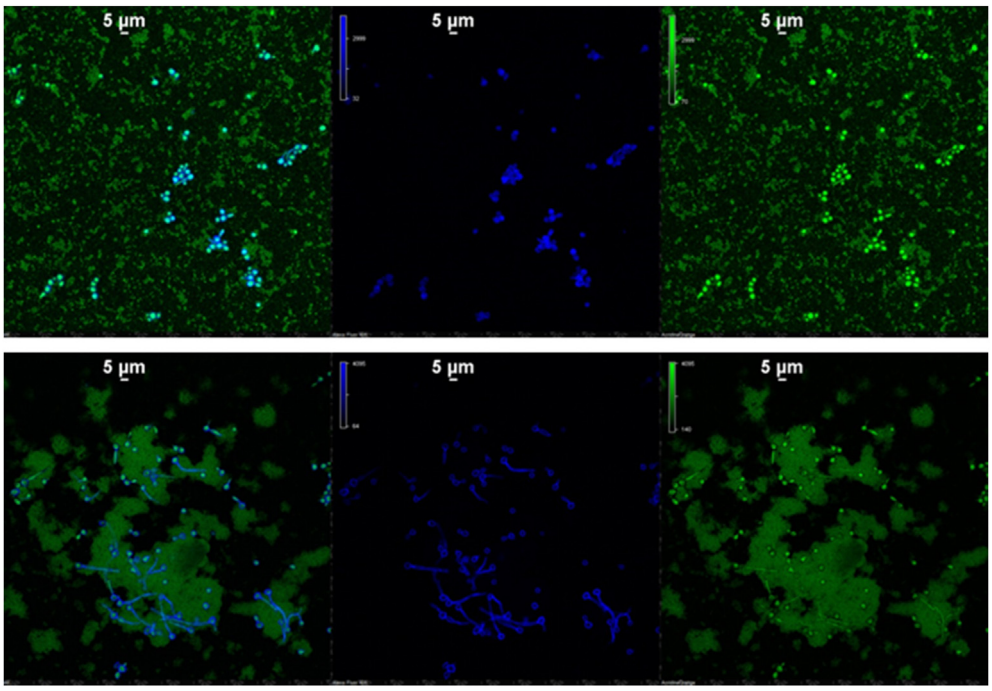

Fig. 7. CLSM images of polymicrobial biofilms with Calcofluor white and SYTO9 collecting fluorescence at 450 nm (blue images) and 525 nm (green) together with a merged image. (Bottom) untreated biofilm, (Top) biofilm treated with the antimicrobials **1** and **2**. LUT intensity scale is displayed for each monochromatic image. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

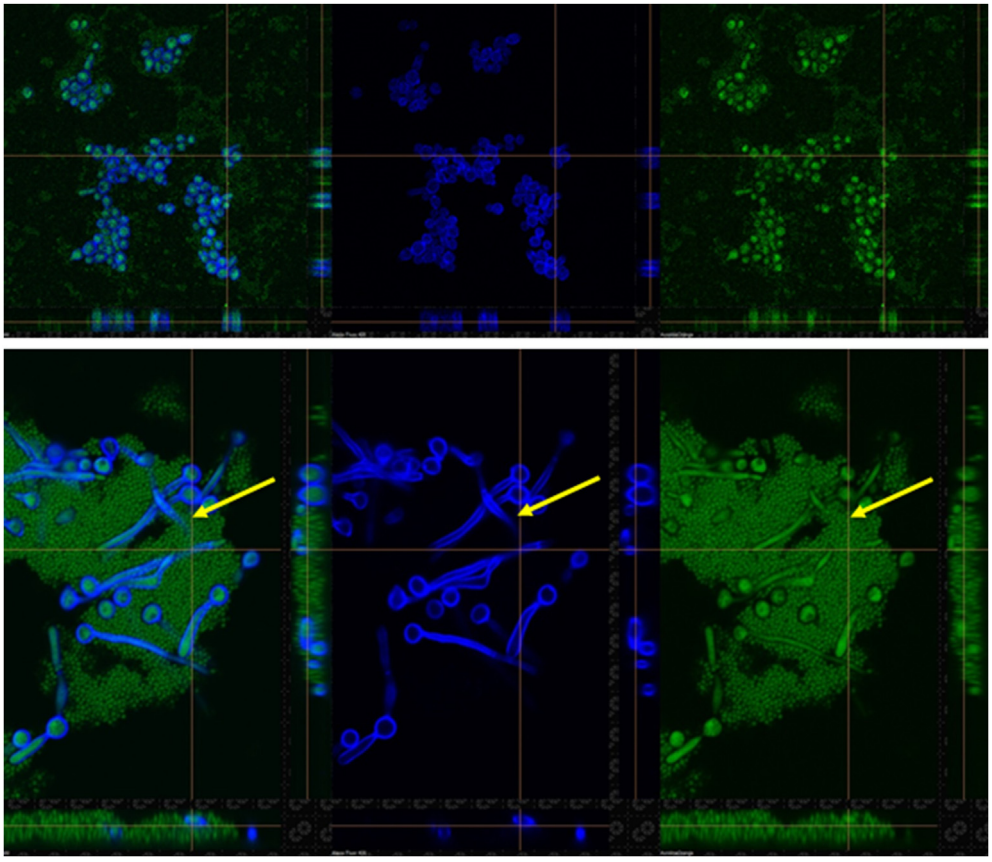

Fig. 8. CLSM Z-stack images of polymicrobial biofilms colored with Calcofluor white and SYTO9 collecting fluorescence at 450 nm (blue images) and 525 nm (green). The yellow arrow in the central panel showing the XY plane for the untreated sample points to evident attachments of staphylococci to the fungal hyphae. The panels on the right and below represent the orthogonal views of the Z-stack displaying the XZ and YZ planes in correspondence of the orange lines. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

chalcone-derivative **1**. Images revealed also a reduction in the number *S. aureus* bacteria.

4. Conclusions

Taken together, the results herein obtained indicate as the combination therapy comprising an antifungal agent and an antibacterial agent could represent a very useful strategy for achieving prevention and treatment of a polymicrobial biofilm when the compounds act in synergy. This strategy embodies an interesting principle to keep on exploring new combinations. A campaign has been undertaken on this prototype combination aimed at optimizing its antimicrobial profile with specific attention on selecting a more effective antifungal agent. A further approach to achieve this goal could be the design and synthesis of hybrid molecules, as hybridization is regarded as a valuable strategy to obtain drug candidates endowed with a multipotent profile. Thus, incorporating the two studied molecules or their pharmacophore units into a single chemical entity offers promises to obtain potent antibacterial and antifungal agents endowed with enhanced anti-biofilm properties. In a medicinal chemistry perspective, this may represent a very challenging task, requiring the hybrid molecule the preservation of a balanced good activity on really different microorganisms. Declaration of competing interest The Authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resmic.2021.103873.

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