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Artemisinins, New Miconazole Potentiators Resulting in Increased Activity against *Candida albicans* Biofilms

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Mucosal biofilm-related fungal infections are very common, and the incidence of recurrent oral and vulvovaginal candidiasis is significant. As resistance to azoles (the preferred treatment) is occurring, we aimed at identifying compounds that increase the activity of miconazole against *Candida albicans* biofilms. We screened 1,600 compounds of a drug-repositioning library in combination with a subinhibitory concentration of miconazole. Synergy between the best identified potentiators and miconazole was characterized by checkerboard analyses and fractional inhibitory concentration indices. Hexachlorophene, pyrvinium pamoate, and artesunate act synergistically with miconazole in affecting *C. albicans* biofilms. Synergy was most pronounced for artesunate and structural homologues thereof. No synergistic effect could be observed between artesunate and fluconazole, caspofungin, or amphotericin B. Our data reveal enhancement of the antibiofilm activity of miconazole by artesunate, pointing to potential combination therapy consisting of miconazole and artesunate to treat *C. albicans* biofilm-related infections.

Multiple fungal species possess the capacity to form biofilms characterized by increased resistance against commonly used antimycotics on both biotic and abiotic surfaces (1, 2). The population of people susceptible to this type of infection is growing, mainly as a consequence of an extended life span, increasing numbers of immunocompromised individuals, and use of indwelling medical devices, which can serve as a substrate for biofilm formation (3–6). Therefore, the occurrence of biofilm-associated infections has expanded over the last several decades, and the extent to which they impact the health of human hosts is enormous (7, 8).

The genus *Candida* predominates in this type of fungal infection occurring in the oral cavity, upper and lower airways, and gastrointestinal and urinary tracts on wounds and medical devices. Such a *Candida* biofilm infection can be of a rather restricted superficial mucosal type or can evolve into hazardous invasive candidiasis (1, 7, 9). Mucosal fungal infections are very common and can often be treated adequately using azoles. However, the incidence of recurrent oral and vulvovaginal candidiasis is significant, and resistance to azoles is occurring (10–13). Vaginal infections caused by *Candida* spp. affect 70 to 75% of women at least once during their lives, and 40 to 50% of them experience at least one recurrence (14). Also, immunocompromised persons, like HIV patients, are susceptible to this type of recurrent candidiasis, mostly involving the oral cavity (15, 16).

Mechanisms underlying the increased resistance of biofilm cells to antimycotics are still not fully understood. However, it has been reported that biofilm formation typically induces several stress response pathways that impair the activity of azole drugs, such as the induction of drug efflux pumps (17). Consequently, cells in a biofilm are up to 1,000-fold more azole resistant than their planktonic counterparts (1, 18), supporting the need for new treatments.

Despite the considerable impact on human health and the problems with resistance related to fungal biofilms, the antimicrobial drug pipeline contains few novel agents that can be used against such biofilm-related infections (19). One approach to overcome the need for new antifungal and antibiofilm compounds is to enhance the activity of existing antimycotics by combining them with another compound, a strategy termed "potentiation." Such so-called potentiators can have multiple modes of action, including the inhibition of tolerance pathways in the biofilm or induction of increased uptake of the antimycotics.

In this study, we employed the concept of repurposing/repositioning existing market drugs. This concept has also recently gained a lot of attention in anti-*Candida* research (20–25). Repurposing of known drugs is favorable from an economic perspective. As these molecules have a safe toxicity profile and dosing regimens are known, the cost of performing new clinical trials and possibly reformulating the drug are considerably less than those for the development of a new drug from scratch (26). For example, toremifene citrate (a selective estrogen receptor modulator used in the treatment of breast cancer) has been reported to be a good potentiator of amphotericin B and caspofungin, but not of azole-type antifungals, against *Candida albicans* biofilms (20). Potentiation of azole antifungals by 2-adamantanamine, a derivative of amantadine (an anti-influenza A virus drug also used to treat some of the symptoms of Parkinson's disease), against *C. albicans* biofilms was recently demonstrated, suggesting the opportunity

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to repurpose (analogues of) other FDA-approved medications (22).

For the above-mentioned reasons, we opted to screen a repositioning-compound library for compounds that can potentiate the activity of the azole miconazole against *C. albicans* biofilms. This concept of potentiation is often favored over the application of single compounds, as it may lead to (i) a widened spectrum of drug activity, (ii) a more rapid antifungal effect, (iii) synergy, (iv) lowered dosing of toxic drugs, and (v) reduced risk of antifungal resistance (27).

MATERIALS AND METHODS

Strains and chemicals. The *C. albicans* strain SC5314 (28) used in this study was grown routinely on YPD (1% yeast extract, 2% peptone [International Medical Products, Belgium], and 2% glucose [Sigma-Aldrich, USA]) agar plates at 30°C. Stock solutions of miconazole (Sigma-Aldrich) were prepared in dimethyl sulfoxide (DMSO) (VWR International, Belgium). RPMI 1640 medium (pH 7.0) with L-glutamine and without sodium bicarbonate was purchased from Sigma-Aldrich and buffered with MOPS (morpholinepropanesulfonic acid) (Sigma-Aldrich). The Pharmakon 1600 repositioning library (Microsource Discovery Systems, USA) was supplied by the Centre of Drug Design and Discovery (Patrick Chaltin, Katholieke Universiteit Leuven, Belgium). Pyrvinium pamoate (salt hydrate) and hexachlorophene were purchased from Sigma-Aldrich. Artesunate, artemisinin, dihydroartemisinin, and artemether were purchased from TCI Europe (Belgium).

Antibiofilm screening assay. A *C. albicans* SC5314 overnight culture, grown in YPD, was diluted to an optical density (OD) of 0.1 (approximately 10^6 cells/ml) in RPMI medium, and 100 μl of this suspension was added to the wells of a round-bottom microplate (TPP Techno Plastic Products AG, Switzerland) (30, 31). After 1 h of adhesion at 37°C, the medium was aspirated, and the biofilms were washed with 100 μl phosphate-buffered saline (PBS) to remove nonadherent cells, followed by addition of 100 μl RPMI 1640 medium. The biofilms were allowed to grow for 24 h at 37°C. Then, 5 μM miconazole was added in combination with 20 μM a compound from the Pharmakon 1600 library (2 mM stock solution in DMSO) in RPMI, resulting in a 1.1% DMSO background. The biofilms were incubated for an additional 24 h at 37°C. Finally, the biofilms were washed and quantified with Cell-Titer Blue (CTB) (Promega, USA) (32) by adding 100 μl CTB diluted 1/10 in PBS to each well. After 1 h of incubation in the dark at 37°C, fluorescence was measured with a fluorescence spectrometer (Synergy Mx multimode microplate reader; BioTek, USA) at a λ_{ex} of 535 nm and a λ_{em} of 590 nm. The fluorescence values of the samples were corrected by subtracting the average fluorescence value of CTB in uninoculated wells (blank). The percentage of metabolically active biofilm cells was calculated relative to the control treatment (1.1% DMSO). Compounds were considered for retesting when their application in the presence of 5 μM miconazole resulted in less than 60% residual metabolic activity of *C. albicans* biofilm cells compared to the control and when the main reported application was not due to antifungal activity.

BEC-2 determination assay. To determine the biofilm eradication concentration 2 (BEC-2) values (the minimal concentration of the compound that causes a 2-fold decrease in biofilm metabolic activity) for the respective compounds, *C. albicans* SC5314 biofilms were grown in a round-bottom microplate as described above. Then, the biofilms were washed with 100 μl PBS, and 100 μl of a concentration series of the compounds in RPMI was added to the biofilms, resulting in a 0.5% DMSO background. The biofilms were incubated for 24 h at 37°C, after which they were washed and quantified with CTB as described above.

Biofilm checkerboard assay. In order to determine possible synergistic interactions between antifungal agents on one hand and identified potentiators on the other hand against *C. albicans* SC5314, checkerboard analysis was used. *C. albicans* biofilms were grown as described above. A combination of antifungal compound and potentiator, 2-fold diluted across rows and columns of a microplate, respectively, was added (DMSO background, 0.6%). After 24 h of incubation at 37°C, the biofilms were quantified by the CTB method. Synergism was determined by fractional inhibitory concentration index (FICI) calculations (20, 33). The FICI was calculated by the following formula: FICI = $[C(\text{BEC-2}_A)/\text{BEC-2}_A] + [C(\text{BEC-2}_B)/\text{BEC-2}_B]$, in which $C(\text{BEC-2}_A)$ and $C(\text{BEC-2}_B)$ are the BEC-2 values of the antifungal drugs in combination and BEC-2_A and BEC-2_B are the BEC-2 values of antifungal drugs A and B alone. The interaction was defined as synergistic for a FICI value of ≤ 0.5, indifferent for $0.5 < \text{FICI} < 4$, and antagonistic for a FICI value of ≥ 4.0 (33).

Planktonic checkerboard assay. Synergistic action on the growth of planktonic cells was determined by FICI calculations as described above. MIC-2 values (the minimal concentration of the compound that causes a 2-fold reduction of planktonic cell growth) were used instead of BEC-2 values. To determine the MIC-2 values for the respective compounds, we utilized conditions similar to those used by Kaneko et al. (24). Briefly, an overnight culture of *C. albicans* SC5314 was diluted to an optical density of 0.1 in synthetic complete (SC) medium (1% complete amino acid supplement mixture [CSM] [MP Biomedicals, USA], 1% yeast nitrogen base [YNB], 2% glucose [Sigma-Aldrich]) in combination with a concentration series of the compounds 2-fold diluted across the rows of the microplate. After 24 h of growth in the presence of the compounds at 37°C, growth was quantified by measuring the OD at 490 nm. The percent growth reduction was calculated relative to the control treatment (0.5% DMSO). In the checkerboard assay, a combination of antifungal compound and potentiator, 2-fold diluted across rows and columns of a microplate, respectively, was added (DMSO background, 0.6%) to the diluted overnight culture. After 24 h of incubation at 37°C, the OD was measured at 490 nm.

Reactive oxygen species (ROS) detection assay. *C. albicans* biofilms, grown as described above, were incubated for 2 to 24 h with artesunate, miconazole, or a combination of both compounds in RPMI at 37°C. After washing the biofilm cells with PBS, the biofilms were incubated in the presence of 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) (Invitrogen, USA) in PBS for 1 h at 37°C. Fluorescence was measured at a λ_{ex} of 470 nm and a λ_{em} of 525 nm.

Statistical analysis. The results were analyzed for statistical significance by an unpaired two-tailed Student *t* test. Values were considered to be statistically significant when the *P* value was <0.05.

RESULTS AND DISCUSSION

Screening for potentiators of the antibiofilm activity of miconazole against *C. albicans* biofilms. We screened 1,600 off-patent drugs and other bioactive agents (the Pharmakon 1600 repositioning library) to identify compounds that can enhance the antibiofilm activity of miconazole against mature biofilms. First, we determined the effects of a concentration series of miconazole alone on mature *C. albicans* biofilms and found that the BEC-2 of miconazole is 92.3 ± 13.0 μM (Fig. 1). We opted to add a combination of the library compound (20 μM) and a subantibiofilm concentration of miconazole (5 μM, resulting in 90 to 100% remaining biofilm activity). We identified 8 compounds that resulted in less than 60% residual metabolic activity of the *C. albicans* biofilm cells when applied in the presence of 5 μM miconazole and with a main reported application other than antifungal activity. They are listed in Table 1, along with their known medical applications.

This initial screening strategy did not discriminate between compounds that affect the biofilm on their own and compounds that enhance the antibiofilm activity of miconazole. To discriminate between these two possibilities, we examined the antibiofilm activities of 20 μM these 8 compounds in the presence and absence of 5 μM miconazole. In Table 1 the compounds are sorted

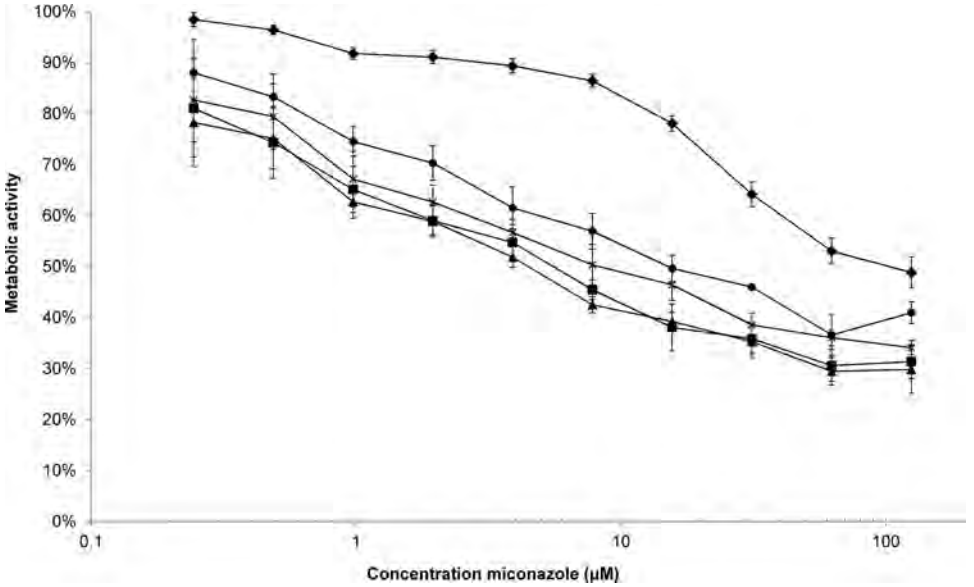

FIG 1 Metabolic activity of *C. albicans* biofilms treated with a combination of artesunate and miconazole (checkerboard assay). The control curve of miconazole alone without artesunate (diamonds) and the curves of combinations of miconazole with 0.625 (circles), 1.25 (crosses), 2.5 (squares), or 5 (triangles) μM artesunate are shown. The values are the means ± standard errors of the mean (SEM) of at least 3 independent biological replicates.

based on the ratio between the residual metabolic activity of the biofilm cells after treatment with the compound in combination with miconazole and that after treatment with the compound alone. For compounds with a ratio of >1, there seems to be an increased antibiofilm effect of the combination with miconazole compared to that of the compound alone. However, many of the top compounds in the list are also very active on their own against *C. albicans* biofilms, as illustrated by their BEC-2 values.

Hexachlorophene, pyrvinium pamoate, and artesunate act synergistically with miconazole to diminish *C. albicans* biofilm activity. The top 3 compounds, based on the ratio between their effects in combination with miconazole and alone (hexachlorophene, pyrvinium pamoate, and artesunate), were selected to determine whether they act synergistically with miconazole against *C. albicans* biofilms. Hexachlorophene is a topical anti-infective drug often used in soaps, liquid detergents, and cosmetics during the 1960s, but its use has been questioned because of its toxicity (34). Pyrvinium pamoate is an antihelmintic drug that, when taken orally, is safe even at high doses, but systemic absorption from the gut is minimal (35). Artesunate, a semisynthetic derivative of artemisinin extracted from *Artemisia annua* (sweet wormwood), is one of the most widely applied antimalarial drugs and is recommended by the World Health Organization (36). We performed checkerboard analysis and calculated the corresponding FICI to determine the synergy (FICI ≤ 0.5) for each of the compounds in combination with miconazole (Table 2). Note that only one strain was used in this study, and clinical strains may behave differently.

TABLE 1 Hits from the miconazole potentiator screen as well as the BEC-2 value of each compound alone is indicated

Compound	Application[a]	Ratio of effects (combination/alone)[b]	BEC-2[c] (μM)
Hexachlorophene	Anti-infective (topical)	2.29	9.3 ± 0.65
Pyrvinium pamoate	Antihelmintic	2.10	3.9 ± 0.13
Artesunate	Antimalarial	1.69	>200
Broxyquinoline	Antiinfectant, disinfectant	1.42	1.2 ± 0.31
Dihydroartemisinin	Antimalarial	1.31	>200
Gentian violet	Antibacterial, antihelmintic	1.18	1.5 ± 0.08
Bithionate disodium	Anthelmintic, antiseptic	1.09	6.0 ± 1.56
Nitroxoline	Antibacterial	0.94	3.1 ± 1.67

[a] As stated in the Pharmakon 1600 information sheet.
[b] The ratio of the antibiofilm effect of 20 μM the repurposed compounds in combination with 5 μM miconazole and alone.
[c] Means ± standard errors of the mean (SEM) of at least 3 independent biological replicates.

Although all three compounds establish a synergistic interaction with miconazole, artesunate is the only potentiator that has almost no antibiofilm activity against *C. albicans* when used alone (according to the BEC-2 value [Table 1]), resulting in the lowest FICI values (Table 2). Additionally, combinations with the compound resulted in the highest reduction of the BEC-2 value of miconazole, as illustrated in Fig. 1 and quantified in Table 2 (fold change). Therefore, the compound was selected for further extensive characterization.

The mechanism of action of artesunate (and structural homologues thereof, collectively called artemisinins) in the treatment of malaria is complex and is only partially understood (37). Much effort is still expended in the elucidation of the modes of action in both *Plasmodium falciparum* (38–40) and yeast (41–43). A yeast model uncovered a role of mitochondria during the action of artemisinins with an important function for the electron transport chain and subsequent damage by locally generated ROS (41). We observed only slightly increased ROS accumulation in *C. albicans* biofilm cells treated with artesunate concentrations starting from 20 μM and a more pronounced ROS accumulation upon incuba-

TABLE 2 Synergistic activities of the potentiators hexachlorophene, pyrvinium pamoate, and artesunate with miconazole against *C. albicans* biofilms

Drug or combination	Potentiator concn (μM)	Miconazole BEC-2 (μM)[a]	*P* value	Fold change[b]	FICI
Miconazole alone		92.3 ± 13.0	NA[c]	NA	NA
Miconazole + hexachlorophene	2.5	9.3 ± 3.4	0.0158	9.9	0.370
	1.25	15.7 ± 6.1	0.0228	5.9	0.305
	0.625	18.5 ± 4.8	0.0265	5.0	0.268
	0.3125	26.0 ± 8.0	0.0397	3.6	0.315
Miconazole + pyrvinium pamoate	1.25	9.3 ± 4.1	0.0164	10.0	0.421
	0.625	18.9 ± 7.7	0.0284	4.9	0.365
	0.3125	28.7 ± 11.6	0.0482	3.2	0.391
Miconazole + artesunate	5	4.0 ± 0.9	0.0050	22.9	<0.069
	2.5	5.2 ± 0.7	0.0055	17.7	<0.069
	1.25	12.2 ± 4.3	0.0194	7.5	<0.139
	0.625	15.2 ± 3.6	0.0234	5.9	<0.171

[a] The BEC-2 values of hexachlorophene, pyrvinium pamoate, and artesunate are 9.3, 3.9, and >200 μM, respectively. The BEC-2 values are the means ± SEM of at least 3 independent biological replicates and were analyzed for statistical significance by an unpaired two-tailed Student *t* test (against the control treatment of miconazole alone).
[b] Fold change, fold increase of biofilm activity of miconazole due to the combination, calculated as follows: (BEC-2 of miconazole alone)/(BEC-2 of miconazole in combination).
[c] NA, not applicable.

tion with miconazole alone (as reported previously [44, 45]), but no increased ROS accumulation due to the combination of both compounds could be observed (data not shown).

Artesunate does not increase the activities of fluconazole, amphotericin B, and caspofungin against *C. albicans* biofilms. We assessed whether artesunate could also increase the activities of other types of azoles, like fluconazole, against *C. albicans* biofilms. Fluconazole showed no significant antibiofilm activity on its own (BEC-2 > 500 μM) or in combination with 20 μM artesunate (BEC-2 > 500 μM).

Kaneko et al. recently showed that artesunate could enhance the activity of the azole fluconazole against planktonic *C. albicans* SC5314 cultures (24). Therefore, we checked whether artesunate could increase the antifungal activity of miconazole or fluconazole on planktonic cells by checkerboard analyses (data not shown). A concentration of 5 μM artesunate reduced the MIC-2 values of miconazole and fluconazole (0.11 ± 0.01 μM and 3.25 ± 0.43 μM, respectively) by 2-fold and 1.6-fold, respectively, in line with the previously reported data (24). However, artesunate did not act synergistically with miconazole or fluconazole on planktonic *C. albicans* cells, as the resulting FICI was higher than 0.5.

Next, we determined whether artesunate could increase the activities of other types of antimycotics, like amphotericin B and caspofungin, against *C. albicans* biofilms (see Fig. S1 and S2 in the supplemental material). However, the BEC-2 values of amphotericin B (1.8 ± 0.4 μM) and caspofungin (0.4 ± 0.06 μM) remained almost unaffected by the presence of all tested concentrations of artesunate, resulting in FICI values between 0.5 and 2. Consequently, the interaction of artesunate with amphotericin B and caspofungin is defined as indifferent.

Structural homologues of artesunate act synergistically with miconazole against *C. albicans* biofilms. Table 1 suggests that a structural homologue of artesunate, namely, dihydroartemisinin, is also capable of increasing the activity of miconazole against *C. albicans* biofilms. Interestingly, one of the compounds that is on the border of the cutoff for the top 8 was another homologue of these compounds, namely, artemisinin (resulting in 61% biofilm metabolic activity in the combination screen [data not shown]). One other structural homologue was available to us during this study, namely, artemether. Via checkerboard analyses, we could

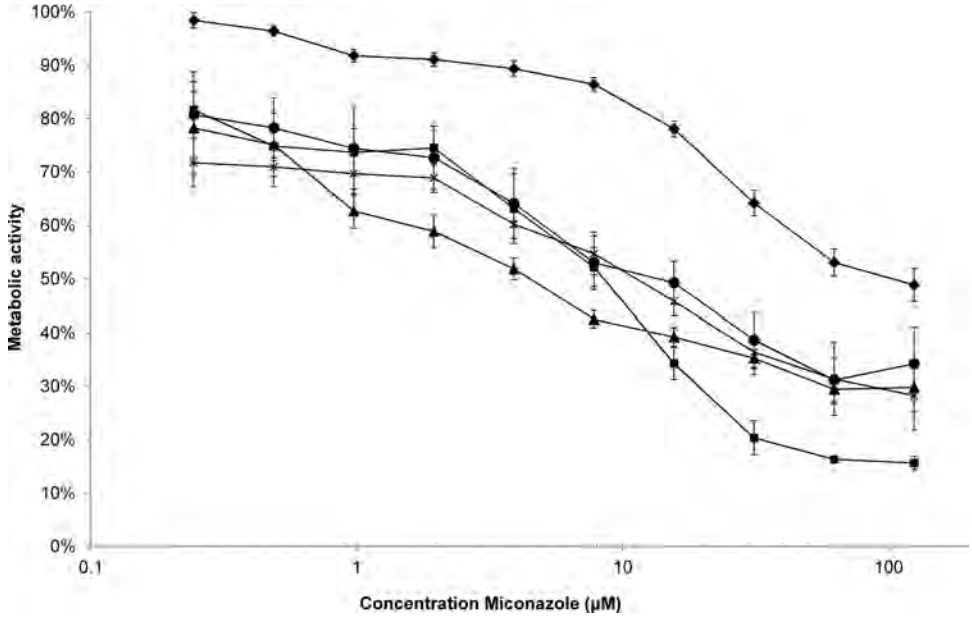

FIG 2 Metabolic activity of *C. albicans* biofilms treated with combinations of miconazole and artesunate, artemisinin, dihydroartemisinin, or artemether. The control curve of miconazole alone (diamonds) and the curves of miconazole with 5 μM artesunate (triangles), artemisinin (circles), dihydroartemisinin (crosses), or artemether (squares) are shown. The values are the means ± SEM of at least 3 independent biological replicates.

show that the observed synergy between artesunate and miconazole against *C. albicans* biofilms is not specific only to artesunate, but also applies to artemisinin, dihydroartemisinin, and artemether (collectively called artemisinin derivatives or artemisinins) (the BEC-2 values for all single compounds were >200 μM) (Fig. 2). A concentration of 5 μM of these compounds with miconazole resulted in 6.2-fold, 8.3-fold, and 11.4-fold reductions in the BEC-2 value of miconazole (resulting in maximal FICI values of 0.186, 0.145, and 0.112) for artemisinin, dihydroartemisinin, and artemether, respectively. These data suggest that synergistic action with miconazole is a characteristic of all artemisinins, implying that the activity is probably attributable to the core chemical structure (a sesquiterpene scaffold with an endoperoxide bridge) of this family.

We conclude that combinations of miconazole with artesunate or other artemisinins could be a novel therapeutic strategy to treat *C. albicans* biofilm-related infections.

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