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Repurposing as a means to increase the activity of amphotericin B and caspofungin against *Candida albicans* biofilms

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Objectives: Biofilms of *Candida* species, often formed on medical devices, are generally resistant to currently available antifungal drugs. The aim of this study was to identify compounds that increase the activity of amphotericin B and caspofungin, commonly used antifungal agents, against *Candida* biofilms.

Methods: A library containing off-patent drugs was screened for compounds, termed enhancers, that increase the *in vitro* activity of amphotericin B against *Candida albicans* biofilms. Biofilms were grown in 96-well plates and growth was determined by the cell titre blue assay. Synergy between identified enhancers and antifungal agents was further characterized *in vitro* using fractional inhibitory concentration index (FICI) values and *in vivo* using a worm biofilm infection model. In light of the application of these enhancers onto implants, their possible effect on the growth potential of MG63 osteoblast-like cells was assessed.

Results: Pre-incubation of *C. albicans* biofilms with subinhibitory concentrations of the enhancers drospirenone, perhexiline maleate or toremifene citrate significantly increased the activity of amphotericin B or caspofungin (FICI <0.5) against *C. albicans* and *Candida glabrata* biofilms. Moreover, these enhancers did not affect the growth potential of osteoblasts. Interestingly, toremifene citrate also enhanced the *in vitro* activity of caspofungin in a mixed biofilm consisting of *C. albicans* and *Staphylococcus epidermidis*. Furthermore, we demonstrate synergy between toremifene citrate and caspofungin in an *in vivo* worm *C. albicans* biofilm infection model.

Conclusions: Our data demonstrate an *in vitro* and *in vivo* enhancement of the antibiofilm activity of caspofungin by toremifene citrate. Furthermore, our results pave the way for implant-related applications of the identified enhancers.

Keywords: yeast, antifungal agents, drug synergy, toremifene citrate

Introduction

Candida albicans and *Candida glabrata* are opportunistic human fungal pathogens that cause not only superficial infections, but also life-threatening systemic diseases. *C. albicans* is the fourth most common cause of bloodstream infections in the USA[1] and has a high attributable mortality rate.[2] *C. glabrata* is an emerging fungal pathogen,[3] with an intrinsic resistance to commonly used antifungal agents.[4,5] *Candida* species often form biofilms on medical devices.[6] Biofilms are structured communities of bacterial and/or fungal cells attached to an inert or biological surface and embedded in a self-produced polymer matrix.[7] These biofilms have great significance for public health, as biofilm-associated infections are frequently refractory to conventional antimicrobial agents. Currently, most antifungal agents are unable to treat these infections effectively, requiring the removal of the device to cure the infection. Liposomal formulations of amphotericin B and the echinocandins are among the only antifungal compounds that display effective antibiofilm activity against *C. albicans* biofilms.[8] Hence, adequate treatment options are limited and new compounds with potent antibiofilm activity are urgently needed.

Apart from the identification of novel antibiofilm molecules with a novel mode of action, an alternative approach to developing effective antibiofilm therapy is to focus on the enhancement of

known antifungal compounds against biofilms. We used the latter approach and screened a repositioning library consisting of off-patent drugs for compounds, termed enhancers, that increase the susceptibility of biofilms to amphotericin B, resulting in an increased activity of amphotericin B against *C. albicans* biofilms. In this study, enhancers are defined as compounds that can increase the antibiofilm activity of an antifungal agent in a concentration range without antifungal or antibiofilm activity. Drug repositioning can accelerate the drug development process as these compounds are characterized by known safety profiles, pharmacology and administration routes.[9,10] Furthermore, molecules that enhance the activity of conventional antifungal agents against biofilms could be used as a coating for medical devices, resulting in improved treatment with conventional drugs in cases of a biofilm-associated device infection. Moreover, by applying these enhancers as implant coatings, the molecules will be available locally at the site of potential biofilm formation and do not need to be supplied systemically through the whole body.

We found that the contraceptive drospirenone, the anti-anginal perhexiline maleate (hereafter referred to as perhexiline) and the anticancer agent toremifene citrate (hereafter referred to as toremifene) can increase the antibiofilm activity of amphotericin B and caspofungin against *C. albicans* and *C. glabrata*, without adverse effects on osteoblast-like cells. Furthermore, we selected toremifene to translate these *in vitro* data to an *in vivo Caenorhabditis elegans* biofilm infection model for *C. albicans*. The antifungal activity of tamoxifen, a close analogue of toremifene, has already been described in the literature.[11–16] However, neither the antibiofilm activity nor the synergy of tamoxifen or toremifene with conventional antifungal agents against biofilm formation or planktonic cultures of *C. albicans* has been described before. An antifungal activity of perhexiline against planktonic cultures of *Cryptococcus neoformans* and *C. albicans* was recently reported;[17] however, an antibiofilm activity of perhexiline or a synergy with conventional antifungal agents has not been described before.

Materials and methods

Strains and chemicals

The strains *C. albicans* CAIF-100,[18] SC5314[19] and *C. glabrata* BG2[20] were used in this study. *Candida* and *Staphylococcus epidermidis* strains were grown routinely on YPD (1% yeast extract, 2% peptone and 2% glucose) and trypticase soy agar [TSA; containing 3% trypticase soy broth (TSB)] plates at 30°C for 2 days, respectively. Stock solutions of amphotericin B (Sigma, St Louis, MO, USA) and caspofungin (Cancidas; Merck, Beeston, Nottingham, UK) were prepared in DMSO. RPMI 1640 medium (pH 7.0) with L-glutamine and without sodium bicarbonate was purchased from Sigma and buffered with MOPS (Sigma). The Pharmakon 1600 repositioning library (MicroSource Discovery Systems, Gaylordsville, CT, USA) was supplied by CD3 (Leuven, Belgium). Drospirenone (6β,7β,15β,16β-dimethylene-3-oxo-17α-pregn-4-ene-21,17-carbolactone), toremifene citrate (2-{4-[(1Z)-4-chloro-1,2-diphenyl-but-1-en-1-yl]phenoxy}-*N*,*N*-dimethylethanamine) and perhexiline maleate [2-(2,2-dicyclohexylethyl)piperidine] were purchased from Sigma.

Antibiofilm screening assay

The Pharmakon 1600 repositioning library was screened in the presence of a sub-biofilm inhibiting concentration 50 (BIC_{50}) amphotericin B concentration, namely 0.156 μM (which results in a 100% survival of *C. albicans* biofilm cells), against *C. albicans* biofilms. The BIC_{50} is the minimal concentration of the compound that inhibits biofilm formation by 50%. To this end, a *C. albicans* CAIF-100 overnight culture, grown in YPD, was diluted to an optical density of 0.1 (~10^6 cells/mL) in RPMI 1640 medium and 95 μL of this suspension was added to the wells of a round-bottomed microtitre plate (TPP, Tradingen, Switzerland) in the presence of 200 μM of each compound (10 mM stock solution in DMSO), resulting in a 2% DMSO background. Biofilms were allowed to grow for 24 h at 37°C. Afterwards, 0.156 μM of amphotericin B was added (final DMSO background 2.1%). The biofilms were incubated for an additional 24 h at 37°C. Finally, the biofilms were washed and quantified with cell titre blue (CTB)[21] by adding 100 μL of CTB diluted 1/10 in PBS to each well. After 1 h of incubation in the dark at 37°C, the fluorescence was measured with a fluorescence spectrometer at an excitation wavelength of 535 nm and an emission wavelength of 590 nm. The fluorescence values of the samples were corrected by subtracting the average fluorescence value of the CTB of uninoculated wells (blank). The percentage of surviving biofilm cells was calculated relative to the control treatment (2.1% DMSO).

Chequerboard antibiofilm assay

In order to determine possible synergistic interactions between the antifungal agents amphotericin B or caspofungin on one hand and drospirenone, perhexiline or toremifene on the other hand against *C. albicans*/*C. glabrata* biofilms, chequerboard analysis was used and fractional inhibitory concentration index (FICI) values were calculated. The FICI was calculated by the formula $FICI = [C(BIC_{50A})/BIC_{50A}] + [C(BIC_{50B})/BIC_{50B}]$, in which $C(BIC_{50A})$ and $C(BIC_{50B})$ are the BIC_{50} of the antifungal drugs in combination, and BIC_{50A} and BIC_{50B} are the BIC_{50} of antifungal drugs A and B alone. The interaction was defined as synergistic for a value of $FICI \leq 0.5$, indifferent for $0.5 < FICI < 4$ and antagonistic for $FICI > 4.0$.[22] To this end, overnight cultures of *C. albicans* SC5314 or *C. glabrata* BG2 were diluted to an optical density of 0.1 in RPMI 1640 medium. Drospirenone (100–3.125 μM), perhexiline (25–0.78 μM) and toremifene (12.5–0.39 μM) were 2-fold diluted across the columns of a round-bottomed 96-well plate in RPMI 1640 medium (TPP, Tradingen Switzerland). Volumes of 5 μL of these compound solutions and 95 μL of the above cell suspension were added to all the wells (DMSO background of 0.5%). After 1 h of adhesion at 37°C, the medium was aspirated and the biofilms were washed with 100 μL PBS to remove non-adherent cells; this was followed by the addition of 100 μL RPMI 1640 medium containing the corresponding compound concentrations. Note that the concentration series of the enhancers that was used did not affect biofilm development by *C. albicans* or *C. glabrata*.

After 24 h of biofilm formation in the presence of the compounds at 37°C, the biofilms were washed with 100 μL PBS, and 100 μL of a combination of amphotericin B or caspofungin and the compounds, 2-fold diluted in RPMI 1640 medium across the rows and columns of a microtitre plate, respectively, was added (final DMSO background 0.6%). The following range was used for amphotericin B: 5–0.01 μM for *C. albicans* and 20–0.04 μM for *C. glabrata*. For caspofungin, 1.25–0.002 μM was used for *C. albicans* and 20–0.04 μM was used for *C. glabrata*. Note that increased concentrations of amphotericin B and caspofungin were used against biofilms of *C. glabrata* as biofilm cells of *C. glabrata* are less susceptible to amphotericin B and caspofungin than biofilm cells of *C. albicans*.

After an additional 24 h of incubation at 37°C, the biofilms of *C. albicans* were quantified with the CTB method as described above. Biofilms of *C. glabrata* were quantified with the XTT assay[23] as *C. glabrata* was not able to convert CTB within 1 h. To this end, biofilms of *C. glabrata* were washed with 100 μL of PBS, and afterwards 100 μL of XTT (0.25 mg/mL in PBS, 1 μM menadione; Sigma, St Louis, MO, USA) was added to every well. After 1 h of incubation at 37°C, the absorbance was measured at 490 nm. The values obtained were corrected for the blank (XTT without cells). All the assays were repeated at least three times, and the average FICI value of at least three independent experiments is shown.

MIC chequerboard assay

MIC tests were performed according to the CLSI protocol M27-A3 in RPMI 1640 medium and 0.6% DMSO background.[24]

Cytotoxicity assay

A cytotoxicity test of the identified enhancers drospirenone, perhexiline and toremifene was performed on a cell type relevant to bone homeostasis, with the aim of screening for enhancer concentrations that did not inhibit cell growth or induce cell death. MG63 osteoblast-like cells, a human osteosarcoma cell line, were obtained from ATCC (American Type Culture Collection CRL-1427; LGC Standards, Molsheim, France). Cells were plated in 24-well plates at 2000 cells/cm^2 in Minimum Essential Medium Eagle—Alpha Modification (αMEM; Sigma, Bornem, Belgium) with 0.292 g/L L-glutamine (G7513; Sigma, Bornem, Belgium) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria) and 1% antibiotic-antimycotic (Gibco® 15240, Life Technologies SAS, Saint Aubin, France). Cells were maintained overnight at 37°C in a humidified environment with 5% CO_2. The media were changed every 48 h.

At day 3 post-seeding, cells were incubated with drospirenone, perhexiline or toremifene by adding the compounds to the culture medium. As a control, a suspension of the same cell line under the same conditions, but without chemicals, was cultured. Two-fold serial dilution assays of the enhancers drospirenone, perhexiline or toremifene were used, starting from 400 μM, 100 μM and 50 μM, respectively. The proliferation of the MG63 cells in the presence or absence of the enhancers was investigated by measuring the total DNA content after 1, 3, 4, 6, 8 and 10 days of incubation, corresponding to 1, 3, 5 and 7 days of enhancer addition. Enhancer concentrations were freshly prepared at each timepoint of addition. Cell proliferation was quantified by determination of the total DNA content. The experiment was performed in duplicate. At each timepoint, the cells were washed twice with PBS and 200 μL of lysis buffer (100 mM Na_2CO_3, 100 mM $NaHCO_3$ and 1 mM $MgCl_2$) was added. All procedures were carried out on ice. Cell lysates were stored at −80°C until further analysis.

For one (out of the two) experiment, viable/dead cells were visualized by using the Live/Dead Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, Carlsbad, CA, USA) prior to adding the lysis buffer. Fluorochromes were added and incubated for 4 min. Subsequently, the reagents were removed and cells were analysed under a fluorescence microscope (Olympus, Tokyo, Japan) at 494 nm for calcein and 528 nm for ethidium homodimer-1. After imaging, the cells were rinsed three times with PBS and lysis buffer was added. The double-stranded DNA content of the lysates was analysed using a Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Frederick, USA) according to the manufacturer's instructions using a microplate reader (Infinite 200; TECAN, Männedorf, Switzerland) at an emission/excitation wavelength of 480/520 nm. The DNA content was determined from measured fluorescence intensities and plotted against the calibration curve for a DNA concentration range of 0 to 1 mg/L. The proliferative responses were presented relative to the day the compounds were added.

Mixed biofilm assay

Enhancement of caspofungin activity by toremifene was tested against mixed species biofilms, consisting of both *C. albicans* and *S. epidermidis*. To this end, overnight cultures of *C. albicans* (YPD) and *S. epidermidis* (TSB) were diluted to a final cell suspension of 5×10^6 cells/mL and 1×10^7cells/mL in RPMI 1640 medium (pH 7.0), respectively. Equal volumes of these cell suspensions of each organism were mixed before use. During biofilm formation (24 h at 37°C), the biofilms were grown in the presence of 6.25 μM toremifene or 0.5% DMSO. After 24 h, biofilms were washed with PBS and treated with 6.25 μM toremifene or 0.3–0.075 μM caspofungin alone or 6.25 μM toremifene in combination with 0.3–0.075 μM caspofungin. DMSO 0.6% served as a negative control. After incubation for 48 h at 37°C, the biofilms were rinsed with PBS, sonicated for 10 min and further detached by thoroughly pipetting up and down. Finally, the biofilm cells were diluted in PBS and plated out on YPD agar plates containing 100 mg/L ampicillin and TSA plates containing 25 mg/L amphotericin B, to determine the number of fungal and bacterial cfu after 2 days of incubation at 37°C, respectively. The percentage of *C. albicans* and *S. epidermidis* cells was determined relative to the DMSO control treatment.

Membrane permeability assay

The induction of membrane permeabilization by toremifene on *C. albicans* biofilm cells was determined using propidium iodide staining (Sigma). To this end, biofilms were grown in RPMI 1640 in the presence (50–1.56 μM) or absence (0.5% DMSO) of toremifene for 24 h. Afterwards, propidium iodide staining was performed as previously described.[25]

Worm infection assay

In vivo experiments using the *C. elegans*/*C. albicans* model system were based on the procedure previously described,[26,27] with minor modifications. Briefly, larvae of *glp-4Δ*/*sek-1Δ* mutants of *C. elegans* were grown to the L4 stage on nematode growth medium (NGM) agar plates containing a surface lawn of freshly inoculated OP50 *Escherichia coli*. Worms were collected, washed with M9 buffer and incubated for 2 h on YPD agar plates containing freshly grown surface lawns of *C. albicans* SC5314. Afterwards, worms were collected and washed with M9 buffer to remove *C. albicans* from their cuticles. Forty to 50 worms were then suspended in 250 μL of M9 buffer (supplemented with 10 mg/L cholesterol, 100 mg/L kanamycin and 75 mg/L ampicillin) containing different drug combinations in separate wells of 24-well plates, and their survival was monitored regularly for 7 days. Worms were treated with 6.25 μM toremifene, 0.095 μM caspofungin, 6.25 μM toremifene + 0.095 μM caspofungin and 0.6% DMSO (negative control). As a control, the survival of non-infected worms was also monitored. Worm survival was expressed as a percentage of their viability at day zero. The data shown represent the mean and standard error of the mean of three independent experiments with six replicates per condition.

Statistical analysis

Results were analysed for statistical significance by the unpaired Student's *t*-test. Values were considered to be statistically significant when the *P* value was <0.05.

Results

Drospirenone, perhexiline and toremifene increase the activity of amphotericin B against *C. albicans* and *C. glabrata* biofilms

We screened 1600 off-patent drugs and other bioactive agents (Pharmakon 1600 repositioning library) to identify compounds that could enhance the antibiofilm activity of amphotericin B, termed enhancers. We opted to include the compounds during the biofilm formation phase in view of a putative application of the enhancers as antibiofilm implant coatings. In cases of potential biofilm formation on an implant coated with the enhancers, the biofilms should become more susceptible to antifungal treatment. A similar strategy was used in a previously published report.[25] We identified 50 compounds that resulted in <10% surviving *C. albicans* biofilm cells in the presence of 0.156 μM amphotericin B. Only nine of these compounds were not characterized as antimicrobial compounds and were selected for further research.

This initial screening strategy did not discriminate between compounds that inhibit growth or biofilm formation on their own or compounds that only enhance the antibiofilm activity of amphotericin B. To discriminate between these two hypotheses, we next determined the potential antibiofilm activity of these nine compounds in the absence of amphotericin B (Table 1). Seven of these compounds displayed antibiofilm activity, as their BIC_{50} values were <100 μM.

Next, we assessed the effect of these nine compounds on the antibiofilm activity of amphotericin B. To this end, *C. albicans* biofilms were incubated for 24 h with a subinhibitory concentration of the compounds, i.e. the highest concentration that did not affect biofilm development, during adhesion and biofilm formation. The resulting biofilms were subsequently incubated with the compounds and a concentration series of amphotericin B, to determine the BIC_{50} of amphotericin B in the presence of the compounds. From these nine compounds, only drospirenone, perhexiline and toremifene were able to increase the antibiofilm activity of amphotericin B against *C. albicans* biofilms by at least 1.5-fold (data not shown). These three potential enhancers were selected for further extensive characterization, as described below.

Table 1. Identified hits and corresponding BIC_{50} values against *C. albicans* biofilms

Compound	BIC_{50} (μM)
Prochlorperazine edisylate	5.2
Danthron	12
Chlorprothixene hydrochloride	17
Toremifene citrate	19.5
Clorgiline hydrochloride	24
Perhexiline maleate	39
Dicyclomine hydrochloride	60
Acamprosate calcium	>100
Drospirenone	400

To determine whether the enhancers drospirenone, perhexiline and toremifene act synergistically with amphotericin B against *C. albicans* biofilms, we calculated the corresponding FICI for each combination by chequerboard analysis (Table 2). The BIC_{50} for drospirenone, perhexiline and toremifene alone was 400, 39 and 19.5 μM against *C. albicans* biofilms. We found that only drospirenone acted synergistically with amphotericin B against *C. albicans* biofilms (FICI ≤ 0.5 for amphotericin B in combination with 50 μM drospirenone). Drospirenone (100–25 μM) reduced the BIC_{50} of amphotericin B 3.8- to 2.2-fold (Table 2). Although the FICI for the other combinations is >0.5, several concentrations of toremifene significantly reduced the BIC_{50} of amphotericin B ($P<0.05$; Table 2). For example, the BIC_{50} of amphotericin B was 3.7-fold reduced in combination with 6.25 μM toremifene (Table 2).

Next, we assessed whether these compounds could also increase the activity of amphotericin B against *C. glabrata* biofilms. The BIC_{50} for drospirenone, perhexiline and toremifene alone

Table 2. Synergistic activity of the enhancers drospirenone, perhexiline and toremifene with amphotericin B or caspofungin against *C. albicans* and *C. glabrata* biofilms

		C. albicans				*C. glabrata*			
AFA + enhancer	Concentration of enhancer (μM)	BIC_{50} AFA (μM) ± SEM	*P*	fold change	FICI	BIC_{50} AFA (μM) ± SEM	*P*	fold change	FICI
AMB + enhancer									
None	—	1.01 ± 0.09	NA	NA	NA	3.89 ± 0.41	NA	NA	NA
Drospirenone	100	0.27 ± 0.02	0.001	3.8	0.514	0.43 ± 0.06	0.001	9.1	0.360
	50	0.32 ± 0.04	0.002	3.2	0.438	1.17 ± 0.28	0.004	3.3	0.425
	25	0.46 ± 0.02	0.007	2.2	0.517	1.33 ± 0.27	0.006	2.9	0.405
Perhexiline	12.5	0.65 ± 0.16	0.075	1.5	0.967	1.11 ± 0.35	0.004	3.5	0.494
	6.25	0.91 ± 0.22	0.623	1.1	1.060	2.1 ± 0.21	0.031	1.9	0.644
	3.125	0.96 ± 0.24	0.811	1.1	1.030	2.43 ± 0.27	0.069	1.6	0.678
Toremifene	6.25	0.27 ± 0.02	0.001	3.7	0.588	1.23 ± 0.35	0.005	3.2	0.540
	3.125	0.49 ± 0.05	0.010	2.0	0.648	2.67 ± 0.58	0.144	1.46	0.798
	1.56	0.60 ± 0.05	0.031	1.7	0.677	2.33 ± 0.38	0.059	1.7	0.656
CAS + enhancer									
None	—	0.29 ± 0.05	NA	NA	NA	12.2 ± 2.24	NA	NA	NA
Drospirenone	100	0.07 ± 0.04	0.024	4.0	0.499	1.96 ± 0.82	0.016	6.2	0.411
	50	0.08 ± 0.04	0.029	3.4	0.416	2.13 ± 1.04	0.033	5.7	0.299
	25	0.12 ± 0.05	0.076	2.4	0.488	3.05 ± 1.19	0.030	4.0	0.312
Perhexiline	12.5	0.04 ± 0.02	0.010	6.5	0.475	0.44 ± 0.07	0.014	27.9	0.244
	6.25	0.08 ± 0.05	0.032	3.4	0.454	0.81 ± 0.16	0.017	15.2	0.170
	3.125	0.09 ± 0.05	0.045	3.1	0.407	1.73 ± 0.08	0.026	7.1	0.193
Toremifene	6.25	0.013 ± 0.01	0.011	21.4	0.367	0.50 ± 0.06	0.007	24.2	0.264
	3.125	0.04 ± 0.02	0.019	7.8	0.289	2.34 ± 1.19	0.021	5.2	0.303
	1.56	0.06 ± 0.03	0.030	5.1	0.278	2.16 ± 0.46	0.018	5.7	0.233

AMB, amphotericin B; NA, not applicable; CAS, caspofungin; AFA, antifungal agent.

was >400, 60 and 28 μM against *C. glabrata* biofilms. To calculate the FICI of drospirenone in combination with caspofungin against *C. glabrata* biofilms, we used a sub-BIC_{50} concentration of 400 μM for drospirenone as higher concentrations of drospirenone could not be used due to their restricted solubility. Drospirenone and perhexiline acted synergistically with amphotericin B against *C. glabrata* biofilms (FICI ≤ 0.5; Table 2). The BIC_{50} of amphotericin B (3.89 μM) in combination with these enhancers was reduced by 9.1- and 3.5-fold, respectively (Table 2). Whereas toremifene did not act synergistically with amphotericin B (FICI > 0.5), 6.25 μM toremifene significantly reduced the BIC_{50} of amphotericin B ($P < 0.05$).

Drospirenone, perhexiline and toremifene act synergistically with caspofungin against C. albicans and C. glabrata biofilms

Putative synergies between drospirenone, perhexiline and toremifene and other commonly used antifungal agents such as caspofungin and fluconazole were investigated against *C. albicans* and *C. glabrata* biofilms. The three compounds did not have a significant effect on the antibiofilm activity of fluconazole (data not shown). However, in contrast to amphotericin B, drospirenone, perhexiline and toremifene all acted synergistically with caspofungin (FICI ≤ 0.5) against biofilms of *C. albicans* and *C. glabrata* grown in presence of the compounds (Table 2). In *C. albicans*, the strongest enhancement of caspofungin activity was observed with toremifene. The BIC_{50} of caspofungin against *C. albicans* biofilms (0.29 μM) was reduced 21.4-fold in the presence of 6.25 μM toremifene. In addition, drospirenone and perhexiline reduced the BIC_{50} of caspofungin by 4- and 6.5-fold, respectively (Table 2). In contrast to *C. albicans*, perhexiline was the strongest enhancer of caspofungin activity against *C. glabrata*; the BIC_{50} (12.2 μM) of caspofungin was reduced by 27.9-fold (Table 2). Drospirenone and toremifene were able to reduce the BIC_{50} of caspofungin against *C. glabrata* biofilms by 6.2- and 24.2-fold, respectively (Table 2).

Furthermore, the effect of drospirenone, perhexiline and toremifene with caspofungin on mature *C. albicans* biofilms was also investigated without pre-treatment of the biofilms with drospirenone, perhexiline and toremifene during adhesion and biofilm formation. In this set-up, only toremifene still acted synergistically with caspofungin in a concentration range of 12.5–3.125 μM (FICI ≤ 0.5). The BIC_{50} of toremifene on mature biofilms was 80 μM. Up to a 6.4-fold reduction in the BIC_{50} of caspofungin was achieved (from 0.29 μM to 0.045 μM in combination with 12.5 μM toremifene). Furthermore, chequerboard analysis with these caspofungin enhancers was also performed on planktonic cells of *C. albicans*. No synergistic effects (FICI > 0.5) were observed in this planktonic set-up, indicating biofilm-specific synergistic effects of drospirenone, perhexiline and toremifene with caspofungin (data not shown).

Differential effect of the enhancers on the viability and growth potential of osteoblast-like cells

Osseointegration is crucial for the fixation of implants into bone and osteoblasts are key players in this process. Therefore, in view of a potential application of these enhancers as implant coatings, we examined the cytotoxic effects of drospirenone, perhexiline and toremifene on MG63 osteoblast-type cells (Figure 1). At day 3 post-seeding, cells were incubated with drospirenone, perhexiline or toremifene by adding different concentrations of the compounds to the culture medium. To compare the toxicity of the compound as a function of the various applied concentrations for each compound, the DNA content was measured after 4, 6, 8 and 10 days of incubation and normalized with respect to the value acquired before addition of the compound (day 3 measurement). For drospirenone, concentrations of 50 μM and lower displayed no cytotoxicity on the human osteoblast-like cells. Upon treatment with perhexiline up to 6.25 μM, human osteoblast-like cells survived and cell proliferation was permitted.

Fifty or 25 μM toremifene was toxic for the MG63 cells, whereas results obtained with lower toremifene concentrations until day 7 post-addition showed no cytotoxicity or inhibition of growth. Live/dead staining of the MG63 cells treated with drospirenone (400–50 μM), perhexiline (100–6.25 μM) and toremifene (50–6.25 μM) confirmed the results obtained from measurements of DNA content. The enhancer perhexiline (100–25 μM) induced cell death, as red-stained nuclei were observed at these concentrations. Drospirenone as well as toremifene seemed to preferentially affect MG63 cell growth and morphology rather than inducing immediate cell death (data not shown). In conclusion, concentrations of toremifene that significantly enhance the action of caspofungin did not affect the growth potential of osteoblast-like cells. In contrast, drospirenone and perhexiline clearly affected the growth potential of osteoblast-type cells at 100 and 12.5 μM, respectively. Based on the *in vitro* and toxicity data described above, we selected toremifene to conduct further experiments.

Toremifene enhances caspofungin activity against mixed biofilms consisting of C. albicans and S. epidermidis

Mixed species biofilms are clinically relevant as in nature most biofilms consist of different yeast and/or bacterial species, and nosocomial *C. albicans* bloodstream infections are often polymicrobial.[28] Moreover, an interaction between fungal and bacterial species in a mixed biofilm environment can influence the virulence and the susceptibility to specific antibiotics of the species.[29–33] Therefore, we investigated whether an enhancement of caspofungin activity by toremifene would also occur in mixed yeast–bacterial biofilms of *C. albicans* and *S. epidermidis*. Mixed biofilms of *C. albicans* and *S. epidermidis* were grown in presence of 6.25 μM toremifene and treated with 6.25 μM toremifene or 0.3–0.075 μM caspofungin alone or a combination of 6.25 μM toremifene with 0.3–0.075 μM caspofungin (Figure 2). Toremifene, caspofungin or a combination of the two compounds had no activity against the bacterial species of the mixed biofilm. However, 6.25 μM toremifene significantly enhanced the activity of 0.15 and 0.075 μM caspofungin against the *C. albicans* cells of the mixed biofilm. At lower or higher caspofungin concentrations, toremifene could not enhance the activity of caspofungin. These data demonstrate that the presence of *S. epidermidis* did not influence the ability of toremifene to increase the activity of caspofungin against *C. albicans* present in a mixed biofilm.

Biofilm-specific synergy between toremifene and caspofungin is independent of membrane permeabilization

Our results suggest a biofilm-specific effect of toremifene on the antibiofilm activity of caspofungin. We hypothesized that

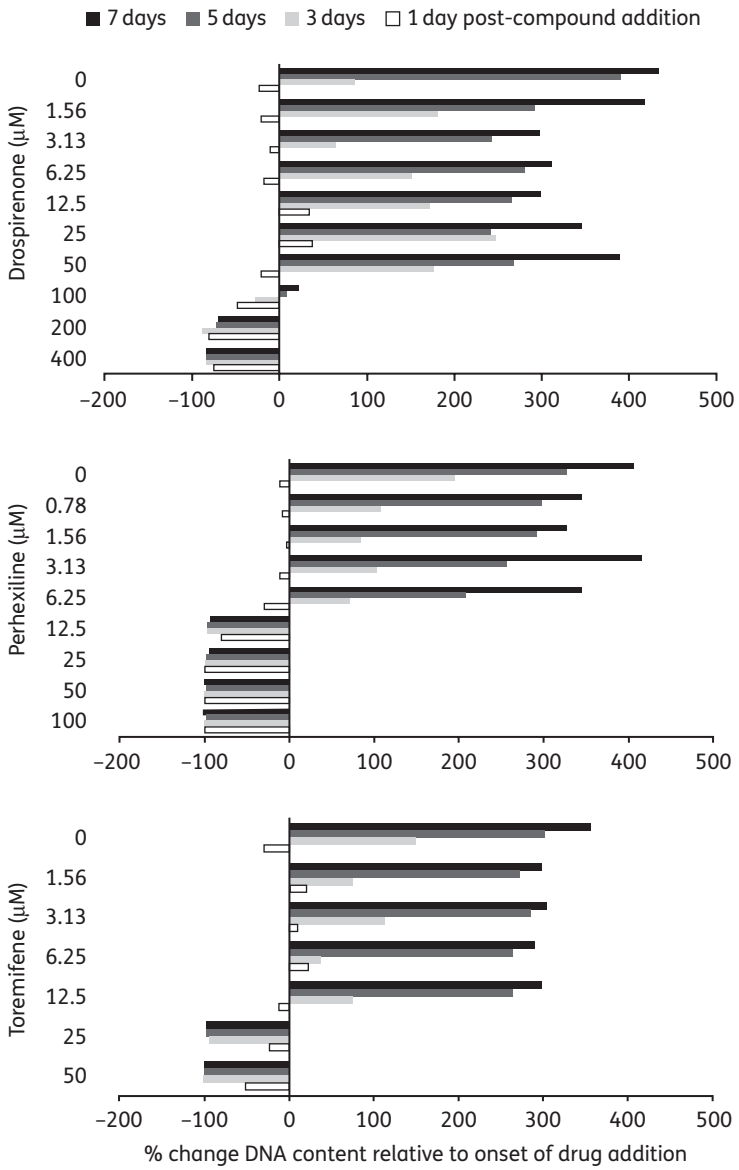

Figure 1. Differential effect of the enhancers on the viability and growth potential of osteoblast-like cells. MG63 cells were grown in 24-well plates in αMEM for 3 days. At day 3 post-seeding, the cells were incubated with drospirenone, perhexiline or toremifene by adding the compounds to the culture medium. As a control, a suspension of this cell line under the same conditions, but without chemicals, was used. MG63 proliferation profiles were evaluated using measurements of DNA content relative to the day the enhancers were added, with measurements after 1, 3, 5 and 7 days of compound addition. This figure represents two independent biological experiments consisting of two technical repeats each.

toremifene might induce membrane permeabilization in *C. albicans* biofilm cells. Using the fluorescent dye propidium iodide, we found that toremifene significantly increased membrane permeabilization at higher concentrations (50–12.5 μM). However, no membrane permeabilization occurred upon incubation with concentrations of 6.25–1.56 μM, which are in the synergistic range for caspofungin (Figure 3). These data demonstrate that the synergistic enhancement of the antibiofilm activity of caspofungin by toremifene seems to be independent of the induction of membrane permeabilization by toremifene, which occurs at higher concentrations.

Toremifene enhances caspofungin activity in a C. albicans worm infection assay

To translate these *in vitro* findings to an *in vivo* infection model, we used the *C. elegans* infection assay,[26] which is regarded as a good infection model for studying biofilm-associated infections.[26,34–38] We selected the most potent combination against *C. albicans* biofilms, which was toremifene/caspofungin, based on the *in vitro* and toxicity data (Table 2 and Figure 1). We used a concentration of caspofungin (0.095 μM) that had only a modest effect on the survival of the infected *C. elegans* worms, with less than one-third of

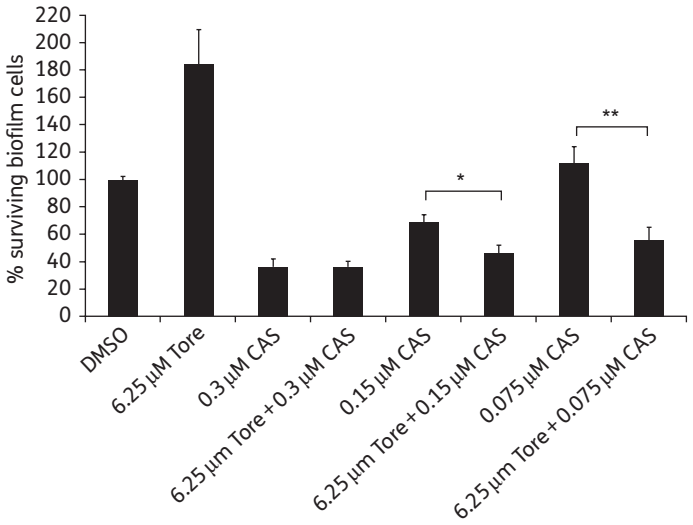

Figure 2. Toremifene enhances the activity of caspofungin against mixed-species biofilms of *C. albicans* and *S. epidermidis*. Mixed-species biofilms of *C. albicans* and *S. epidermidis* were grown in RPMI 1640 medium in the presence or absence of 6.25 μM toremifene for 24 h at 37°C. After 24 h, the biofilms were washed and treated with 6.25 μM toremifene or 0.3–0.075 μM caspofungin alone or a combination of toremifene and caspofungin. After 48 h, the percentage survival was quantified by plating the biofilm cells on YPD agar plates containing 100 mg/L ampicillin and TSA plates containing 25 mg/L amphotericin B. As toremifene, caspofungin and a combination of the two had no effect on the survival of *S. epidermidis* present in the mixed biofilm, only the percentage of surviving *C. albicans* biofilm cells relative to the control treatment (0.6% DMSO) is shown. The data represent the mean and standard error of the mean of three independent experiments, consisting of three technical repeats each. Tore, toremifene; CAS, caspofungin. $^{*}P<0.05$; $^{**}P<0.01$.

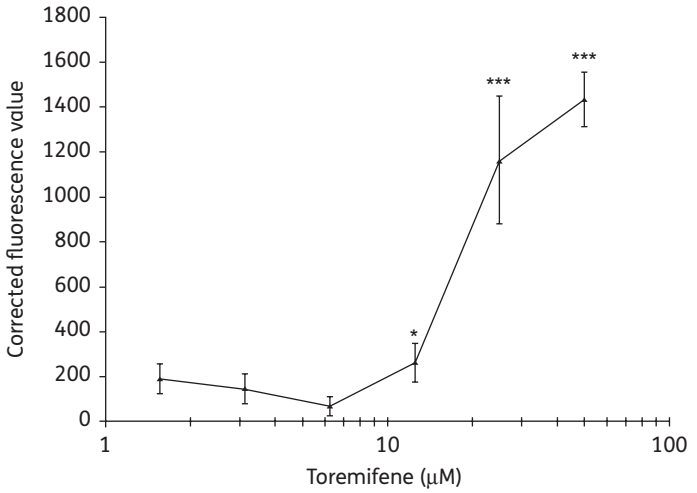

Figure 3. Toremifene induces membrane permeabilization in *C. albicans* biofilm cells. *C. albicans* biofilms were grown in RPMI 1640 medium in the presence (50–1.56 μM) or absence (0.5% DMSO) of toremifene for 24 h. After 24 h, the biofilms were washed and membrane perme-abilization was measured using the fluorescent dye propidium iodide. The fluorescence values are corrected to those of untreated biofilms (0.5% DMSO). The data presented are the mean and standard error of the mean of three independent biological repeats, each consisting of at least three measurements. $^{*}P<0.05$; $^{***}P<0.001$.

the worms surviving after 7 days. Infected worms were treated with 6.25 μM toremifene or 0.095 μM caspofungin alone, or with a combination of 6.25 μM toremifene and 0.095 μM caspofungin (Figure 4). Treatment of the infected worms with a combination of 6.25 μM toremifene and 0.095 μM caspofungin significantly increased the survival of the worms compared with treatment with 6.25 μM toremifene or 0.095 μM caspofungin alone or control treatment (0.6% DMSO) at 3, 5, 6 and 7 days post-infection ($P<0.001$). Seven days post-infection, $57.08\pm3.09\%$ of the worms were still surviving when treated with the combination of caspofungin and toremifene. In contrast, treatment with caspofungin or toremifene alone resulted in only $30.99\pm2.09\%$ or $17.99\pm2.92\%$ surviving worms, respectively, whereas only $13.5\pm2.28\%$ of the worms treated with 0.6% DMSO (control treatment) survived after 7 days (Figure 4). The above data indicate that toremifene also acts synergistically with caspofungin in the *in vivo C. elegans* infection model. As there was no significant difference between treatments with toremifene alone and with the control (0.6% DMSO), it seems that 6.25 μM toremifene produces no toxic side effects in the nematodes (Figure 4). The lack of toxicity of toremifene on the worms corroborates our previous findings regarding the growth potential of the osteoblasts, which is unaffected by toremifene up to concentrations of 12.5 μM.

Discussion

Biofilms are critical in the development of clinical infections of pathogenic fungi such as *C. albicans* and *C. glabrata*.[39] As these biofilms are resistant to almost all the currently available antifungal agents, new antifungal drugs with antibiofilm activity and new therapeutic concepts are urgently needed. In the search for such new molecules, two main strategies can be followed: screening for (i) novel antibiofilm molecules characterized by a biofilm-specific mode of action, or (ii) molecules that enhance the activity of antifungal agents such as amphotericin B, caspofungin and fluconazole against biofilms. An enhancement of the activity of existing antifungal agents against biofilms will allow a lowering of their effective dose and thus reduce potential toxic side effects and economic costs. The use of repositioning libraries in this respect has the advantage that the toxicological and pharmacological properties of the different compounds are known and, consequently, promising molecules can be rapidly translated into clinical use.[10]

In this study, we screened a repositioning library for compounds that enhance the activity of amphotericin B against *C. albicans* biofilms grown in presence of the compounds. We identified three compounds, i.e. enhancers, that increased the activity of amphotericin B and caspofungin against biofilms of *C. albicans* and *C. glabrata*. Chequerboard analysis revealed synergistic activity for drospirenone, perhexiline and toremifene with caspofungin against biofilms of *C. albicans* and *C. glabrata* ($FICI\leq0.5$). In several combinations, up to a 20-fold reduction of the caspofungin concentration necessary to inhibit biofilm formation by 50% (BIC_{50}) of *C. albicans* or *C. glabrata* was achieved. Moreover, we identified a biofilm-specific enhancement of caspofungin activity as no synergy was observed in planktonic conditions. These data indicate that the enhancement of activity of an antifungal

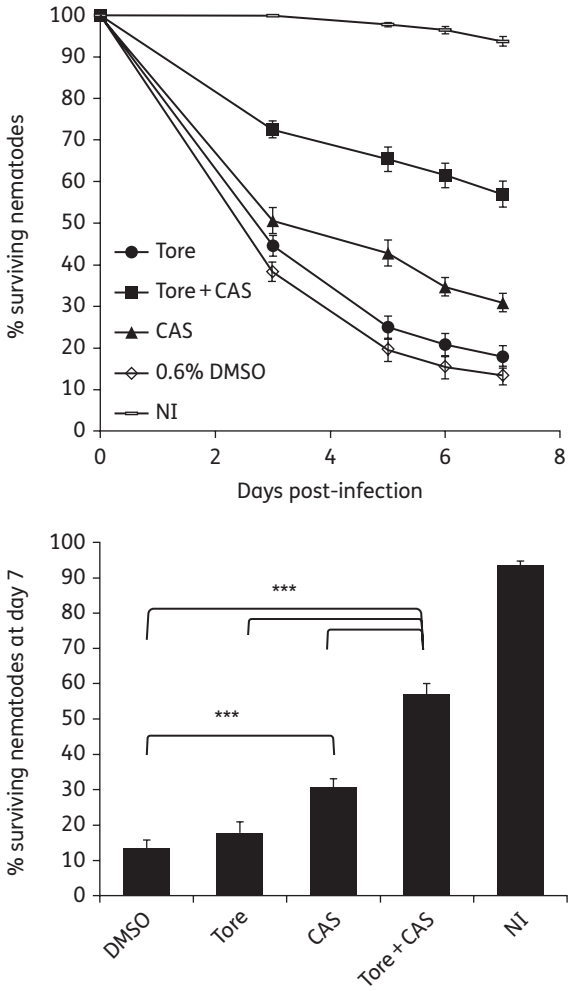

Figure 4. Toremifene acts synergistically with caspofungin in a *C. elegans* infection assay. *C. elegans* worms were infected with *C. albicans* by feeding them on an YPD plate containing *C. albicans* for 2 h. Afterwards, the worms were treated with 6.25 μM toremifene, 0.095 μM caspofungin or 6.25 μM toremifene + 0.095 μM caspofungin. Untreated worms and non-infected worms served as controls with a DMSO background of 0.6%. The worms were counted regularly for 7 days post-infection. Worm survival was expressed as a percentage of the worms' viability at day 0. The data presented are the mean and standard error of the mean of three independent biological repeats, each consisting of six technical repeats per condition. NI, non-infected worms; Tore, toremifene; CAS, caspofungin. ***$P < 0.001$.

compound can be biofilm-specific. Although treatment with toremifene and caspofungin did not affect *S. epidermidis* in the mixed biofilm assay, toremifene was still able to enhance the activity of caspofungin against *C. albicans* in a mixed biofilm set-up. This may be clinically relevant as the presence of *C. albicans* in a mixed biofilm environment can influence the virulence and the susceptibility to specific antibiotics of the bacterial species and, conversely, *S. epidermidis* can lower the susceptibility of *C. albicans* to antifungal agents.[29–31,40] Furthermore, we translated these *in vitro* results of toremifene regarding an enhancement of caspofungin activity to a *C. elegans* biofilm infection model for *C. albicans*. The treatment of infected worms with a combination of toremifene and caspofungin resulted in a significant increased survival of the *C. elegans* worms compared with a single treatment with toremifene, caspofungin or DMSO (control treatment).

A similar screening was performed by LaFleur *et al*.[41] They screened an NIH repositioning library for enhancers of the azole antifungal clotrimazole against biofilms of *C. albicans*. Different hits were identified compared with our study. The amphotericin B/caspofungin enhancers that we identified could not enhance the activity of fluconazole, pointing toward a different mode of action of azole enhancers versus amphotericin B/caspofungin enhancers for *Candida* biofilms.

Few reports describe caspofungin enhancers against *Candida* biofilms based on a synergistic activity as substantiated by a FICI ≤ 0.5. We have previously demonstrated that the non-steroidal anti-inflammatory drug diclofenac acts synergistically with caspofungin *in vitro* and *in vivo* in a catheter-associated biofilm rat model.[25] Furthermore, synergy was also demonstrated for the calcineurin inhibitor cyclosporine A and caspofungin. Both developing and mature biofilms were more susceptible to caspofungin in combination with cyclosporine A compared with treatment with caspofungin alone.[42] Recently, synergistic activity of the antibiotic colistin with caspofungin has also been shown against planktonic *C. albicans* cultures.[43]

The three identified compounds in this study are well characterized for their applications in other medical domains. Drospirenone is a synthetic hormone used in several birth control pills in combination with ethinylestradiol.[44] Drospirenone is also approved by the FDA to treat premenstrual dysphoric disorder and moderate acne vulgaris as reviewed by Fenton *et al*.[45] Perhexiline has been used clinically as an anti-anginal agent for over 25 years.[46] Finally, toremifene is a selective oestrogen receptor modulator, which binds to oestrogen receptors.[47] Toremifene is used in the treatment of oestrogen receptor-positive breast cancer and is approved for treatment of this type of cancer in several countries.[48] Furthermore, toremifene shows promising results in preventing prostate cancer.[49,50] Interestingly, other selective oestrogen receptor modulators, namely tamoxifen and clomiphene, have been identified as enhancers of fluconazole against planktonic cultures of the yeast *Saccharomyces cerevisiae*, whereas they act only in an additive way with fluconazole against *C. albicans* planktonic cells.[9] Thus, synergy of tamoxifen with conventional antifungal agents was tested only on planktonic *C. albicans* cells, showing no synergistic interaction. In this study, we demonstrate a biofilm-specific synergistic interaction of the tamoxifen analogue toremifene with the echinocandin caspofungin against biofilms of *C. albicans* and *C. glabrata*. The selective oestrogen receptor modulators tamoxifen, toremifene and clomiphene are triphenylethylenes.[51] The structure of tamoxifen and toremifene differs by a single chloride ion.[52] An antifungal activity of tamoxifen has been reported for >20 years;[11–16] however, to our knowledge, an antibiofilm activity of tamoxifen or toremifene against *C. albicans* biofilms has never been described.

The antifungal activity of tamoxifen is based on its membrane-perturbing effects and an interference with calcium homeostasis and calcineurin signalling.[12,15,16] We tested the putative membrane permeabilization activity of toremifene against *C. albicans* biofilms using the probe propidium iodide. Membrane permeabilization was observed at concentrations ≥12.5 μM, pointing, at least in part, to a mode of antifungal activity similar to that of tamoxifen. However, the concentrations of toremifene used to

enhance caspofungin activity did not induce membrane permeabilization. These data indicate that the toremifene-induced enhancement of caspofungin activity against *C. albicans* biofilms is probably not due to its antifungal mode of action based on membrane permeabilization, but instead affects a biofilm-specific target. Note that, as amphotericin B permeabilizes the fungal membrane, this may increase the amount of compound that enters the cell. However, as we also observed synergistic interactions between caspofungin and these enhancers and caspofungin is not known to permeabilize the fungal membrane, it seems rather unlikely that the observed increased activity of amphotericin B would be solely due to an increased transport of these enhancers into the cell.

Up to a quarter of implants are subject to revision surgery due to infection.[53] Because of the increasing use of medical devices for orthopaedic and dental implants, the burden of implant failure and consecutive surgical revision is expected to increase by >100% over the next 25 years.[54] The formation of bacterial and/or fungal biofilms that are resistant to current antibiotics/antifungal agents is the major factor responsible for implant infections. The new generation of cementless implants contain biocompatible and bioactive porous coatings that, on one hand, enable fast osseointegration of the implant but, on the other hand, result in an increasing risk of microbial contamination due to the high porosity of the surface coating on the implant.[55] To reduce biofilm-associated infections on implants, biocidal coatings can be applied based on (i) the use of metal ions such as silver, which are toxic when they accumulate, or (ii) the release of standard antibiotics/antifungal agents to which biofilms display increasing tolerance. Moreover, such continuous antibiotic/antifungal pressure increases the incidence of clinical drug resistance. Therefore, applications of the currently identified enhancers onto the implant are of great importance in this field. A local application of the enhancers, via for example the coating of an implant or medical device in general, will result in biofilms that, if formed, are more susceptible (up to 20-fold) to conventional standard antifungal agents. Such a strategy could greatly enhance the treatment options for biofilm-associated device infections. In this regard, we tested the enhancers for their effect on the viability and growth potential of osteoblast-like cells. We found that none of the enhancers affected the growth potential of the osteoblasts when applied at a concentration that synergistically enhanced the action of standard antifungal agents against biofilms, thereby paving the way for the local application of such enhancers onto implants or other medical devices. Whereas these enhancers can be used in controlled-release coatings, further research is required to determine the remaining activity of these enhancers when attached, covalently or not, to the implant.

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Transparency declarations

None to declare.

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