Derivatives of the Mouse Cathelicidin-Related Antimicrobial Peptide (CRAMP) Inhibit Fungal and Bacterial Biofilm Formation

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We identified a 26-amino-acid truncated form of the 34-amino-acid cathelicidin-related antimicrobial peptide (CRAMP) in the islets of Langerhans of the murine pancreas. This peptide, P318, shares 67% identity with the LL-37 human antimicrobial peptide. As LL-37 displays antimicrobial and antibiofilm activity, we tested antifungal and antibiofilm activity of P318 against the fungal pathogen *Candida albicans*. P318 shows biofilm-specific activity as it inhibits *C. albicans* biofilm formation at 0.15 μ M without affecting planktonic survival at that concentration. Next, we tested the *C. albicans* biofilm-inhibitory activity of a series of truncated and alanine-substituted derivatives of P318. Based on the biofilm-inhibitory activity of these derivatives and the length of the peptides, we decided to synthesize the shortened alanine-substituted peptide at position 10 (AS10; KLKKIAQKIKNFFQKLVP). AS10 inhibited *C. albicans* biofilm formation at 0.22 μ M and acted synergistically with amphotericin B and caspofungin against mature biofilms. AS10 also inhibited biofilm formation of different bacteria as well as of fungi and bacteria in a mixed biofilm. In addition, AS10 does not affect the viability or functionality of different cell types involved in osseointegration of an implant, pointing to the potential of AS10 for further development as a lead peptide to coat implants.

In natural environments such as the human body, fungal and bacterial species are typically found in biofilms. The latter are well-structured populations of microbial cells attached to a surface and embedded in a self-produced polymer matrix (1, 2). Biofilms can be formed on natural body surfaces or on medical devices, including urinary and vascular catheters, implants, prostheses, and heart valves (3, 4). These biofilms are of great significance for public health, as they are critical in the development of clinical infections and are frequently refractory to conventional antimicrobial agents (5). The cause of this increased resistance is not yet fully understood but could be due to a combination of different mechanisms, including (i) expression of resistance genes, (ii) binding of the antimicrobials to the extracellular matrix, (iii) alteration in microbial membrane composition, and (iv) presence of microbial persister cells, which are cells that are transiently tolerant of high doses of an antimicrobial agent (6).

Most pathogens, including *Candida* species (3), *Pseudomonas aeruginosa*, *Escherichia coli* (7), and *Porphyromonas gingivalis* (8), can cause biofilm-associated infections. Several species of the genus *Candida*, including *C. albicans*, *C. glabrata*, *C. krusei*, and *C. dubliniensis*, are opportunistic human fungal pathogens that may cause life-threatening systemic infections, particularly in immunocompromised patients. *P. aeruginosa* and *E. coli* are important opportunistic bacteria involved in several infections, including persistent airway infections in cystic fibrosis patients and urinary tract infections, respectively (9, 10). The Gram-negative oral anaerobe *P. gingivalis* is involved in the pathogenesis of periodontitis (8).

Most of the currently available antifungals and antibiotics are unable to cure these biofilm-associated infections effectively (11, 12). Therefore, treatment often requires the removal of the infected device, which can be an expensive and painful surgical procedure. Hence, new compounds with potent antibiofilm activity, preferentially active against both fungal and bacterial biofilms, are

urgently needed. Apart from the systemic administration of antibiofilm compounds to cure biofilm-associated infections, such molecules can be used as a coating on, e.g., medical devices, thereby preventing biofilm formation by microbial pathogens on the device and thus resulting in a reduced risk for development of biofilm-associated device infections. Current antibiofilm coatings of medical devices are mainly based on the use of silver ions, which can be toxic to the host upon accumulation (13), or on the release of standard antibiotics or antifungal agents for which biofilms display increased resistance.

Cationic antimicrobial host defense peptides represent a promising class of antimicrobials and are ubiquitous in nature as components of innate immune defense systems (14–16). Moreover, they are widely regarded as a potential source of future antibiotics owing to a remarkable set of advantageous properties ranging from a broad spectrum of activity to a low propensity for resistance development (17). The major human cationic host defense peptide is LL-37 (Table 1). In addition to its key role in modulating the innate immune response and antimicrobial activity, LL-37 potentially inhibits the biofilm formation of several Gram-negative and Gram-positive bacterial species (16, 23–25). Development of *P. aeruginosa* biofilms is inhibited *in vitro* at low (<1 μ g/ml) LL-37 concentrations which are at least 100-fold below the

Received 11 April 2014 Returned for modification 7 May 2014

Accepted 24 June 2014

Published ahead of print 30 June 2014

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doi:10.1128/AAC.03045-14

TABLE 1 LL-37 derivatives and CRAMP derivatives and their antibiofilm activity^a

Peptide	Sequence	AB	Reference or source
LL-37	LLGDFFRKSKEKIGKEF KRIVQRIKD FLRN LVP RTES	+	18
LL-31	LLGDFFRKSKEKIGKEF KRIVQRIKD FLRN L -----	+	18
LL7-31	-----RKSKEKIGKEF KRIVQRIKD FLRN L -----	+	19
LL17-32	----- FKRIVQRIKD FLRN L V-----	ND	20
LL13-37	----- GKEFKRIVQRIKD FLRN LVP RTES	ND	20
KS30	-----KSKEKIGKEF KRIVQRIKD FLRN LVP RTES	+	21
KR20	----- KRIVQRIKD FLRN LVP RTES	+	21
CRAMP	---GLLRKGGEKIGKEKL KKIGQKIKN FFQKL V PQPEQ	ND	22
CRAMP-18	-----GEKL KKIGQKIKN FFQKL-----	ND	22
P318	-----KIGEK LKKIGQKIKN FFQKL V PQPEQ		This study
AS10	-----KL KKTAQKIKN FFQKL V P-----		This study

^a AB, reported antibiofilm activity; +, antibiofilm activity reported in corresponding reference; ND, not determined. Amino acid residues in bold are conserved.

concentration required to kill or inhibit bacterial growth (16). Apparently, LL-37 affects biofilm formation by decreasing the attachment of bacterial cells and by downregulating genes essential for biofilm development (16). A structure-activity analysis of the effect of LL-37 on bacterial biofilm formation showed that peptide fragments of LL-37 are able to inhibit biofilm formation at sub-bactericidal concentrations without displaying cellular toxicity (18, 19). A 25-amino-acid (aa)-long LL-37 derivative, LL7-31 (Table 1), was found to possess antibacterial and antibiofilm activities against *P. aeruginosa* without showing cytotoxicity (19). Further shortening of the LL-37 backbone to a 20-aa fragment resulted in KR20, still exhibiting bacterial antibiofilm activity (21). Apart from antibacterial activity, LL-37, as well as truncated versions LL13-37 and LL17-32, is able to inhibit planktonic growth of the human fungal pathogen *C. albicans*. Wong and colleagues showed that LL13-37 permeabilizes the *Candida* cell membrane and induces the production of reactive oxygen species (20). LL-37 also inhibits adhesion of *C. albicans* to plastics and tissues by interacting with yeast cell wall carbohydrates (26) and elevating the β -1,3-exoglucanase activity of Xog1 (27). All these data point to potential activity of LL-37 against formation of *C. albicans* biofilms, as adhesion is an important step in this process. However, *C. albicans* antibiofilm activity of LL-37 has never been directly demonstrated.

In the present study, we developed a shortened variant of mouse LL-37, termed AS10, which was characterized by potent antibiofilm activity against *C. albicans* and against various bacterial species, including *E. coli*, *P. aeruginosa*, and *P. gingivalis*. In light of its potential application as an antibiofilm coating on medical devices and implants, the absence of cytotoxicity of AS10 and its effect on functionality of human osteoblasts, mesenchymal stromal cells, and endothelial cells were investigated.

MATERIALS AND METHODS

Strains and chemicals. Strains *C. albicans* SC5314 (28), *C. albicans* DAY286 (29), *C. albicans* CAF2-1 (28), *C. glabrata* BG2 (30), *C. krusei* IH6104 (Belgian Co-ordinated Collections of Microorganisms [BCCM], Antwerp, Belgium), *C. dubliniensis* NCPF3949 (31), *Staphylococcus epidermidis* (32), *E. coli* TG1 (33), *P. aeruginosa* PA14 (34), and *P. gingivalis* ATCC 33277 (35) were used in this study. Overnight cultures of *C. albicans* were grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose) at 30°C. Overnight cultures of *S. epidermidis*, *P. aeruginosa*, and *P. gingivalis* were grown in TSB (3% Trypticase soy broth) at 37°C. Overnight cultures of *E. coli* were grown in lysogeny broth (LB) medium at

37°C. RPMI 1640 medium with L-glutamine and without sodium bicarbonate was purchased from Sigma and buffered to pH 7.0 with MOPS (morpholinepropanesulfonic acid; Sigma, St. Louis, MO) (final concentration, 165 mM). Stock solutions of caspofungin acetate (Cancidas; Merck, Beeston Nottingham, United Kingdom) and amphotericin B (Sigma, St. Louis, MO) were prepared in dimethyl sulfoxide (DMSO). Phosphate-buffered saline (PBS) was prepared by combining 8 g liter⁻¹ NaCl, 0.291 g liter⁻¹ KCl, 1.44 g liter⁻¹ Na₂HPO₄, and 0.24 g liter⁻¹ KH₂PO₄ (pH 7.4). All P318-derived peptides were synthesized by Thermo Scientific (Ulm, Germany) at a purity of at least 95% as determined by liquid chromatography (LC) and mass spectrometry (MS). All peptides were stored as a 3 mM stock solution in 70% acetonitrile at -20°C. Suitable aliquots were dried in a Speedvac concentrator (Savant) and were dissolved in MilliQ water before use.

Fungicidal activity against *C. albicans* cells. The MFC-2 (the minimal fungicidal concentration that kills 50% of planktonic *C. albicans* cells relative to the growth control) of AS10 was determined. To this end, 2-fold serial dilution series of AS10 were prepared in MilliQ water and subsequently diluted 10-fold in RPMI 1640. Next, 5- μ l volumes of these dilutions were transferred to the wells of a flat-bottomed 96-well microtiter plate. A 5- μ l volume of MilliQ water was used as a control. Afterward, 95 μ l of an overnight culture of *C. albicans*, diluted to an optical density at 600 nm (OD₆₀₀) of 0.1 in RPMI 1640, was added to the wells. After 24 h of static incubation at 37°C, viability of the cells was assessed by plating on YPD agar plates. Afterward, the number of CFU was counted and the percentage of surviving cells relative to the control treatment was calculated.

Antibiofilm assay: inhibition or eradication of *Candida* biofilms grown on polystyrene microtiter plates. The potential of the peptides for inhibition of *Candida* biofilm formation was assessed using the cell titer blue (CTB) quantification method (36) or the 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) assay (37). To this end, an overnight culture of *Candida* was washed with PBS and a cell suspension of 10⁶ cells/ml was prepared in RPMI 1640 medium (pH 7.0). Two-fold serial dilution series of the peptides (dissolved in MilliQ water) were prepared in RPMI medium. Five-microliter volumes of these series were added with 95 μ l of inoculum to each well of a round-bottomed polystyrene 96-well microtiter plate (TPP, Trasadingen, Switzerland). After 24 h of static incubation at 37°C, biofilms were washed and quantified as described before (38, 39). Activity of AS10 against biofilms of *C. glabrata*, *C. dubliniensis*, and *C. krusei* was assessed with the XTT assay (38, 39). The percentage of fluorescence/absorbance, which correlates with metabolically active biofilm cells, was calculated relative to the control treatment level. BIC-2 and BIC-5 are defined as the minimal concentrations of a compound resulting in 2- and 5-fold decreases in biofilm formation relative to the control treatment level, respectively. Data represent the means and standard errors of the

means (SEM) of the results of at least 2 experiments, each consisting of 2 technical repeats.

Eradication of fungal biofilms was determined using a previously described protocol (39). To this end, 100 μ l of an overnight culture of *C. albicans*, diluted to 10^6 cells/ml ($OD_{600} = 0.1$) in RPMI 1640 medium (pH 7.0), was added to each well of a round-bottom polystyrene 96-well microtiter plate (TPP, Trasadingen, Switzerland). After 1 h of adhesion at 37°C, nonadherent cells were removed and 100 μ l of RPMI 1640 was added to each well. After 24 h of incubation at 37°C, biofilms were washed with PBS. Two-fold serial dilution series of the peptides were prepared in RPMI 1640 and added to the biofilms. After 24 h of static incubation at 37°C, biofilms were rinsed with PBS and quantified with CTB as previously described (38). The percentage of fluorescence, which correlates with metabolically active biofilm cells, was determined relative to the control treatment. The experiment was performed twice, with each experiment consisting of 4 independent technical repeats.

Antibiofilm assay: inhibition of biofilm formation of *C. albicans* on titanium disks. Commercially pure titanium sheets (grade 2; Goodfellow, Huntingdon, United Kingdom) 1 mm in thickness were laser cut into square discs of 0.64 cm². Titanium disks were placed in a 24-well plate (Greiner Bio one, Kremsmünster, Austria), and 500 μ l of a *C. albicans* suspension (10^6 cells/ml) in RPMI 1640 containing 12.5 or 6.25 μ M AS10 or MilliQ water (control treatment) was added to the wells of the disks. After an adhesion phase of 1 h, disks were washed with 500 μ l PBS and placed in a new microtiter plate. To all the wells, 500 μ l of fresh RPMI 1640 medium containing 12.5 or 6.25 μ M AS10 or MilliQ water (0.5% control treatment) was added. After 24 h of biofilm formation, disks were washed twice by the addition of 1 ml PBS to the disks. Biofilms were dissociated from the disks by sonication (10 min) and thorough a vortex procedure (30 s), which was repeated twice. Finally, cell suspensions were diluted and plated on YPD agar plates. After 2 days of incubation at 30°C, CFU were counted. Three biologically independent experiments were performed consisting of duplicate repeats.

Antibiofilm assay: inhibition of bacterial biofilms. A static peg assay for the prevention of bacterial biofilm formation was used as described previously (40, 41). The device used for biofilm formation was a platform carrying 96 polystyrene pegs (Nunc no. 445497) that fits as a microtiter plate lid with a peg hanging into each microtiter plate well (Nunc no. 269789). Two-fold serial dilutions of the compounds in 100 μ l liquid broth (TSB diluted 1/20) per well were prepared in the microtiter plate (2 repeats per compound). Subsequently, an overnight culture of *E. coli* TG1 (grown in LB medium) or *P. aeruginosa* PA14 (grown in TSB) was diluted 1:100 into the respective liquid broth, and 100 μ l ($\sim 10^6$ cells) was added to each well of the microtiter plate, resulting in a total amount of 200 μ l medium per well. *P. gingivalis* (ATCC 33277) overnight cultures were grown anaerobically (Anoxomat, AN20; Mart Microbiology, Drachten, the Netherlands) in TSB at 37°C, and 100 μ l of a 1×10^8 cell suspension in TSB was added to the wells. Thereafter, the pegged lid was placed on the microtiter plate and the plate was incubated for 24 h at 37°C without shaking. Biofilms of *P. gingivalis* were grown anaerobically. During this incubation period, biofilms were formed on the surface of the pegs. For quantification of biofilm formation, the pegs were washed once in 200 μ l PBS. The remaining attached bacteria were stained for 30 min with 200 μ l 0.1% (wt/vol) crystal violet in an isopropanol-methanol-PBS solution (1:1:18 [vol/vol]). Excess stain was rinsed off by placing the pegs in a 96-well plate filled with 200 μ l distilled water per well. After the pegs were air dried (30 min), the dye bound to the adherent cells was extracted with 30% glacial acetic acid (200 μ l per well of a 96-well plate). The OD_{570} of each well was measured using a Synergy MX multimode reader (Biotek, Winooski, VT). The BIC-2 value for each compound was derived from the concentration gradient using nonlinear curve fitting (GraphPad Prism 5; Graphpad Software, Inc., La Jolla, CA). Data represent means $\pm$ SEM.

Mixed biofilm assay. Mixed biofilms of *C. albicans* and *S. epidermidis* were grown and quantified as described previously (38). Briefly, biofilms were grown in the presence or absence of AS10 during both the adhesion

phase and the maturation phase. Afterward, biofilms were quantified using plating on YPD agar plates containing 100 mg/liter ampicillin and TSB agar plates containing 25 mg/liter amphotericin B. The percentage of *C. albicans* and *S. epidermidis* biofilm cells was determined relative to the MilliQ water control treatment.

Checkerboard antibiofilm assay. To determine synergistic interactions between AS10 and the antifungal agents caspofungin, amphotericin B, and miconazole against mature *C. albicans* biofilms, a checkerboard analysis was used and fractional inhibitory concentration index (FICI) values were calculated. The FICI values were calculated by the formula $FICI = [C(BEC-2 A)/BEC-2 A] + [C(BEC-2 B)/BEC-2 B]$, in which $C(BEC-2 A)$ and $C(BEC-2 B)$ are the BEC-2 values of the antifungal agents in combination and BEC-2 A and BEC-2 B are the BEC-2 values of antifungal agents A and B alone. The BEC-2 is the biofilm-eradicating concentration which is the minimal concentration of a compound that results in a 2-fold eradication of the biofilm relative to the control treatment. The interaction was defined as synergistic when $FICI \leq 0.5$, indifferent when $0.5 < FICI < 4$, and antagonistic when $FICI > 4.0$ (42). For these determinations, an overnight culture of *C. albicans* SC5314 was diluted in RPMI 1640 medium to a cell density of 10^6 cells/ml. One hundred microliters of this inoculum was added to the wells of a round-bottom microtiter plate (TPP, Trasadingen, Switzerland). After 1 h of adhesion at 37°C, the medium was aspirated and the biofilms were washed with 100 μ l PBS to remove nonadherent cells, followed by the addition of 100 μ l fresh RPMI 1640 medium. After 24 h of biofilm development at 37°C, the biofilms were washed with 100 μ l PBS, after which 100 μ l of a combination of AS10 with caspofungin (0.01 to 5 μ M), amphotericin B (0.01 to 5 μ M), or miconazole (0.2 to 100 μ M), in each case 2-fold serially diluted in RPMI 1640 medium across the columns and rows of the microtiter plate, respectively, was added to each well (final DMSO background of 0.1%). After 24 h of treatment at 37°C, biofilm mass was quantified with the CTB method as previously described (38). A sub-BEC-2/BEC-5 value of AS10 (400 μ M) was used to calculate the FICI values, as concentrations as high as 400 μ M AS10 did not have any effect on mature biofilms (as opposed to biofilm formation). FICI values for the AS10-caspofungin/amphotericin B interaction of 3 independent experiments are shown.

Mammalian-cell viability assay. Cell viability of three human primary cell cultures, namely, osteoblasts, mesenchymal stromal cells, and microvascular endothelial cells, was tested with trypan blue staining according to the ISO 10993-5 standard, as previously described (39). Briefly, cells were seeded at 2×10^4 cells/cm² in 96-well tissue culture test plates (TPP, Switzerland) and were exposed in 4 replicates to (i) medium, (ii) medium with 0.05% phenol (cytotoxic control), and (iii) medium with AS10 (12.5 μ M). After 6 days of culture, the cell culture medium was removed from the 96 wells and a 1/3 dilution of trypan blue stock (0.4%)—Dulbecco's modified Eagle's medium (DMEM) was added to the culture for 3 min. Dead (blue) and live (transparent) cells were counted in 2 visual fields for each of the four replicates.

Endothelial tube formation assay. Tube formation was determined as previously described (39). Briefly, human aortic endothelial cells were cultured in M200 medium with low-serum growth supplement (all from Gibco, Carlsbad, CA). A 24-well plate was coated with 250 μ l of phenol-red-free Matrigel (Becton, Dickinson, Bedford, MA) per well, and human aortic endothelial cells were seeded at 2×10^4 cells/cm². The medium was supplemented with phenol (0.05%) or AS10 (12.5 μ M). Nonsupplemented medium was used as the control. Eight hours after seeding of the cells, 3 random phase-contrast digital images in each of the 3 wells per treatment were taken. Tube-like structures were analyzed with ImageJ software (<http://rsbweb.nih.gov/ij/>), and average tube length per field was calculated.

Osteogenic differentiation. The effect of peptides on osteogenic differentiation potential was assessed as previously described (39). Briefly, osteoblasts and mesenchymal stromal cells were cultured in osteogenic medium without or with 12.5 μ M AS10. Osteoblast and mesenchymal

stromal cell cultures were harvested after 3 or 5 weeks for the calcium and DNA assay.

Calcium and DNA assay. Calcium deposition of osteoblasts and mesenchymal stromal cells was measured with the Calcium CPC LiquiColor test (Stanbio Laboratory, Boerne, TX) as previously described (39). Briefly, cell cultures were extracted with 5% trichloroacetic acid (500 μ l per sample), O-cresolphthalein complex one was added, and the calcium content was determined spectrophotometrically at 550 nm. DNA content was determined as previously described (39). DNA values were used to normalize calcium content. Four wells per condition were examined, and two samples from each well were taken for each assay.

Statistical analysis. The statistical significance of the results from the above-described cell assays was tested with one-way analysis of variance (ANOVA) followed by Tukey's test. *P* values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Inhibition of *C. albicans* biofilm formation by P318 and P318 sequence variants. The Section of Animal Physiology and Neurobiology (KU Leuven, Belgium) houses a peptide library with more than 1,000 putative bioactive peptides that were identified from different species. P318 (KIGEKLKKIAQKIKNFFAKLVAQPEQ; Table 1), a shortened variant of the known cathelicidin-related antimicrobial peptide (CRAMP; Table 1), was recently discovered in the islets of Langerhans of the murine pancreas (43; Bart Landuyt, Walter Luyten, and Liliane Schoofs, personal communication). As CRAMP shares 67% sequence identity with the LL-37 human antimicrobial peptide (Table 1), we assessed the potential antibiofilm activity of P318 against *C. albicans* using the metabolic dye CTB. P318 concentrations as low as 0.15 μ M decreased biofilm formation of *C. albicans* up to 2-fold (BIC-2).

We subsequently performed a structure-activity relationship study of P318 to identify amino acids important for its antibiofilm activity. Truncated derivatives of P318 as well as a series of variants in which amino acids were individually replaced by alanine were synthesized, and the corresponding BIC-2 and BIC-5 values of these P318 derivatives were determined. P318 was identified in mouse tissue (pancreas) both in unmodified form and in a modified C-terminal-amidated form. As the latter modification is known to influence stability and activity, both forms were tested in this structure-activity relationship. All truncated peptides showed decreased antibiofilm activity compared to P318, although several peptides still had BIC-2s lower than 0.3 μ M (Table 2). For more-stringent selection of the best truncated and sequence-modified derivatives, we used an additional parameter, namely, BIC-5.

Once the peptides reach a certain minimal length, BIC-2 and BIC-5 values of some P318-derived peptides can drastically increase (Table 2). For example, the BIC-2 values of 318_13, 318_38, and 318_51 were significantly increased compared to those of the closely related 318_12, 318_37, and 318_50 peptides, respectively, which are only 1 aa longer. Note that the biofilm-inhibitory activity of 318_4 and 318_28 is more than 30-fold decreased compared to that of the closely related 318 variants which are either 1 aa shorter or longer (Table 2). The reason for the drastically decreased activity of these particular peptides is currently being investigated. In contrast to most of the P318-derived peptides, which can inhibit biofilm formation by 50% and 80% at similar concentrations, peptide 318_35 shows a BIC-2 of 0.30 μ M but a BIC-5 > 25 μ M. Combining BIC-2 and BIC-5 values with the minimal length of a peptide showing *C. albicans* biofilm-inhibi-

tory activity, peptide 318_50, which is 8 aa shorter than P318, has the most potent biofilm-inhibitory activity, with BIC-2 and BIC-5 values of 0.24 μ M and 0.38 μ M, respectively (Table 2).

In parallel, the biofilm-inhibitory activity of variant forms of P318, in which each amino acid of the native sequence was individually replaced by an alanine residue, was determined (Table 3). Replacement of most of the amino acids did not result in substantially altered BIC values. However, replacement of the 1st (318_AS1) or 14th (318_AS14) amino acid with an alanine resulted in antibiofilm activity that was reduced more than 2- or 5-fold, respectively, compared to the native P318 sequence activity (Table 3). These data demonstrate the importance of the replaced lysines, which is in line with data obtained by Shin and colleagues (44). They showed that substitution of non-positively charged residues in CRAMP-18 (Table 1) with lysines resulted in increased antibacterial and antifungal activity against planktonic cells. Replacement of the 5th (318_AS5), 10th (318_AS10), or 21st (318_AS21) amino acid by alanine resulted in BIC-2 $\leq$ 0.120 μ M.

Combining the results obtained in Table 2 and Table 3, we decided to synthesize peptide P318_50_AS10, here referred to as AS10. The sequence of this peptide (KLKKIAQKIKNFFQKLVP) is based on the sequence of peptide 318_50 combined with an alanine replacement of the glycine in position 6 (position 10 in the native P318 sequence). P318_50 was chosen as a template based on its potent antibiofilm activity combined with a minimal length of the peptide (Table 2). We chose to focus only on the alanine substitution of the glycine in position 10 of P318 as the other two alanines (in positions 5 and 21) are at the outer borders of the AS10 sequence (positions 1 and 17). The BIC-2 and BIC-5 values of AS10 are 0.22 μ M and 0.34 μ M, respectively. Thus, by combining the alanine replacement in position 10 with the P318_50 truncated version, we obtained improved BIC-2- and BIC-5 values compared to those of P318_50. Note that the amino acid sequence of AS10 shares 50% identity with the corresponding region of LL-37 (Table 1).

AS10 specifically inhibits *C. albicans* biofilm development. Concentrations of AS10 corresponding to its BIC-2 and BIC-5 values do not affect survival of planktonic *C. albicans* cells (MFC-2 = 50 μ M). This is in line with observed biofilm inhibition of *P. aeruginosa* at low LL-37 concentrations, which are at least 100-fold below the concentration required to kill or inhibit planktonic growth (16). Also, the recently identified OSIP108 plant decapeptide inhibits *C. albicans* biofilm formation at concentrations that do not affect viability of *C. albicans* cells (39). We also checked if AS10 could inhibit biofilm formation of different *Candida* species, including *C. glabrata*, *C. krusei*, and *C. dubliniensis*. However, none of the AS10 concentrations used (BIC-2 > 100 μ M) could prevent biofilm formation of these *Candida* strains, indicating that AS10 displays *C. albicans*-specific antibiofilm activity. To investigate whether the observed antibiofilm activity of AS10 is strain (SC5314) specific, we also tested other *C. albicans* strains such as DAY286 and CAF2-1 (see Table 5). AS10 showed comparable levels of antibiofilm activity against all tested *C. albicans* strains. The BIC-2 values of AS10 against biofilms of DAY286 and CAF2-1 were 0.32 ± 0.05 μ M and 0.67 ± 0.18 μ M, respectively, whereas the BIC-2 of AS10 against SC5314 was 0.22 ± 0.02 . To further investigate whether the AS10 antibiofilm activity is due to interference with the adhesion of planktonic cells to the polystyrene substrate, we performed additional experi-

TABLE 2 Biofilm-inhibitory activity of truncated forms of P318

Peptide	Sequence	BIC-2 (μM) $\pm$ SEM	BIC-5 (μM) $\pm$ SEM
P318	[H] KIGEKLLKIGQKIKNFFQKLVPQPEQ [NH ₂]	0.15 $\pm$ 0.03	0.32 $\pm$ 0.04
318_1	[H] IGEKLLKIGQKIKNFFQKLVPQPEQ [NH ₂]	0.33 $\pm$ 0.10	7.83 $\pm$ 1.86
318_2	[H] GEKLLKIGQKIKNFFQKLVPQPEQ [NH ₂]	0.20 $\pm$ 0.04	0.43 $\pm$ 0.07
318_3	[H] EKLLKIGQKIKNFFQKLVPQPEQ [NH ₂]	0.25 $\pm$ 0.04	0.33 $\pm$ 0.01
318_4	[H] KLLKIGQKIKNFFQKLVPQPEQ [NH ₂]	>25	>25
318_5	[H] LKKIGQKIKNFFQKLVPQPEQ [NH ₂]	0.21 $\pm$ 0.01	0.30 $\pm$ 0.00
318_6	[H] KKGQKIKNFFQKLVPQPEQ [NH ₂]	0.25 $\pm$ 0.03	0.40 $\pm$ 0.03
318_7	[H] KIGQKIKNFFQKLVPQPEQ [NH ₂]	0.24 $\pm$ 0.02	0.35 $\pm$ 0.02
318_8	[H] IGQKIKNFFQKLVPQPEQ [NH ₂]	0.30 $\pm$ 0.04	0.46 $\pm$ 0.06
318_9	[H] GQKIKNFFQKLVPQPEQ [NH ₂]	0.43 $\pm$ 0.13	0.58 $\pm$ 0.14
318_10	[H] QKIKNFFQKLVPQPEQ [NH ₂]	1.50 $\pm$ 0.50	10.30 $\pm$ 3.90
318_11	[H] KIKNFFQKLVPQPEQ [NH ₂]	0.30 $\pm$ 0.04	1.74 $\pm$ 0.73
318_12	[H] IKNFFQKLVPQPEQ [NH ₂]	0.78 $\pm$ 0.25	4.50 $\pm$ 1.50
318_13	[H] KNFFQKLVPQPEQ [NH ₂]	8.37 $\pm$ 4.52	18.33 $\pm$ 1.67
318_14	[H] NFFQKLVPQPEQ [NH ₂]	8.40 $\pm$ 4.18	20.00 $\pm$ 5.00
318_15	[H] FFQKLVPQPEQ [NH ₂]	12.00 $\pm$ 4.16	>25
318_16	[H] FQKLVPQPEQ [NH ₂]	>25	>25
318_17	[H] QKLVPQPEQ [NH ₂]	>25	>25
318_18	[H] KLVPQPEQ [NH ₂]	>25	>25
318_19	[H] LVPQPEQ [NH ₂]	>25	>25
318_20	[H] VPQPEQ [NH ₂]	>25	>25
318_21	[H] PQPEQ [NH ₂]	>25	>25
318_22	[H] QPEQ [NH ₂]	>25	>25
318_23	[H] PEQ [NH ₂]	18.75 $\pm$ 4.27	>25
318_24	[H] KIGEKLLKIGQKIKNFFQKLVPQPE [OH]	0.33 $\pm$ 0.03	0.55 $\pm$ 0.09
318_25	[H] KIGEKLLKIGQKIKNFFQKLVPQP [OH]	0.40 $\pm$ 0.07	0.60 $\pm$ 0.15
318_26	[H] KIGEKLLKIGQKIKNFFQKLVPQ [OH]	0.25 $\pm$ 0.04	0.48 $\pm$ 0.14
318_27	[H] KIGEKLLKIGQKIKNFFQKLVP [OH]	0.28 $\pm$ 0.02	0.31 $\pm$ 0.04
318_28	[H] KIGEKLLKIGQKIKNFFQKL [OH]	10.90 $\pm$ 2.34	13.75 $\pm$ 3.06
318_29	[H] KIGEKLLKIGQKIKNFFQKL [OH]	0.28 $\pm$ 0.04	0.38 $\pm$ 0.01
318_30	[H] KIGEKLLKIGQKIKNFFQK [OH]	0.30 $\pm$ 0.00	1.70 $\pm$ 0.30
318_31	[H] KIGEKLLKIGQKIKNFFQ [OH]	0.23 $\pm$ 0.05	0.47 $\pm$ 0.06
318_32	[H] KIGEKLLKIGQKIKNFF [OH]	0.22 $\pm$ 0.03	0.40 $\pm$ 0.00
318_33	[H] KIGEKLLKIGQKIKNF [OH]	0.28 $\pm$ 0.03	0.59 $\pm$ 0.12
318_34	[H] KIGEKLLKIGQKIKN [OH]	0.29 $\pm$ 0.02	0.53 $\pm$ 0.16
318_35	[H] KIGEKLLKIGQKIK [OH]	0.30 $\pm$ 0.00	>25
318_36	[H] KIGEKLLKIGQKI [OH]	0.44 $\pm$ 0.09	0.70 $\pm$ 0.10
318_37	[H] KIGEKLLKIGQK [OH]	0.36 $\pm$ 0.08	0.80 $\pm$ 0.00
318_38	[H] KIGEKLLKIGQ [OH]	3.50 $\pm$ 1.00	5.83 $\pm$ 0.17
318_39	[H] KIGEKLLKIG [OH]	4.17 $\pm$ 3.71	6.00 $\pm$ 0.00
318_40	[H] KIGEKLLKI [OH]	4.00 $\pm$ 1.15	8.00 $\pm$ 1.50
318_41	[H] KIGEKLLK [OH]	12.38 $\pm$ 4.32	>25
318_42	[H] KIGEKLLK [OH]	>25	>25
318_43	[H] KIGEKLL [OH]	>25	>25
318_44	[H] KIGEKLL [OH]	>25	>25
318_45	[H] KIGEKLL [OH]	>25	>25
318_46	[H] KIGEKLL [OH]	>25	>25
318_47	[H] IGEKLLKIGQKIKNFFQKLVPQPE [OH]	0.28 $\pm$ 0.02	0.33 $\pm$ 0.03
318_48	[H] GEKLLKIGQKIKNFFQKLVPQP [OH]	0.25 $\pm$ 0.03	0.45 $\pm$ 0.05
318_49	[H] EKLLKIGQKIKNFFQKLVPQ [OH]	0.25 $\pm$ 0.05	0.28 $\pm$ 0.05
318_50	[H] KLLKIGQKIKNFFQKLVP [OH]	0.24 $\pm$ 0.06	0.38 $\pm$ 0.08
318_51	[H] LKKIGQKIKNFFQKLVP [OH]	>25	>25

ments (on DAY286) where AS10 was added only after the adhesion phase. The BIC-2 of AS10 in this setup was $0.65 \pm 0.05 \mu\text{M}$. The fact that the BIC-2 in the latter setup increased only 2-fold (compared to $0.32 \mu\text{M}$ when AS10 is present during the adhesion phase) indicates that AS10 interferes mainly with the biofilm formation process rather than with biofilm adherence. Finally, to investigate whether AS10 also inhibits *C. albicans* biofilm formation on substrates other than polystyrene, we investigated the ac-

tivity of AS10 against biofilm formation of *C. albicans* on titanium disks as a model for implant substrates. To this end, *C. albicans* biofilms were grown on titanium disks in the absence or presence of AS10 (12.5 and $6.25 \mu\text{M}$) for 24 h (Fig. 1). Using CFU determination, we found that both concentrations of AS10 can significantly prevent biofilm formation of *C. albicans* on titanium disks ($P < 0.05$). These data indicate that (i) the observed antibiofilm activity of AS10 is not polystyrene specific and (ii) AS10's antib-

TABLE 3 *C. albicans* biofilm-inhibitory activity of P318 sequence variants

Peptide	Sequence	BIC-2 (μM) ± SEM	BIC-5 (μM) ± SEM
318_AS1	[H] AIGELKKIGQKIKNFFQKLVQPPEQ [NH2]	0.36 ± 0.05	0.53 ± 0.08
318_AS2	[H] KAGEKLKKIGQKIKNFFQKLVQPPEQ [NH2]	0.16 ± 0.03	0.24 ± 0.04
318_AS3	[H] KIAELKKIGQKIKNFFQKLVQPPEQ [NH2]	0.15 ± 0.03	0.23 ± 0.03
318_AS4	[H] KIGAKLKKIGQKIKNFFQKLVQPPEQ [NH2]	0.15 ± 0.03	0.24 ± 0.02
318_AS5	[H] KIGEALKKIGQKIKNFFQKLVQPPEQ [NH2]	0.09 ± 0.01	0.16 ± 0.02
318_AS6	[H] KIGEKAKKIGQKIKNFFQKLVQPPEQ [NH2]	0.16 ± 0.03	0.25 ± 0.03
318_AS8	[H] KIGEKLKAIGQKIKNFFQKLVQPPEQ [NH2]	0.15 ± 0.03	0.28 ± 0.03
318_AS9	[H] KIGEKLKKAGQKIKNFFQKLVQPPEQ [NH2]	0.18 ± 0.01	0.25 ± 0.03
318_AS10	[H] KIGEKLKKIAQKIKNFFQKLVQPPEQ [NH2]	0.12 ± 0.02	0.21 ± 0.01
318_AS12	[H] KIGEKLKKIGQAQKIKNFFQKLVQPPEQ [NH2]	0.16 ± 0.01	0.26 ± 0.02
318_AS13	[H] KIGEKLKKIGQKAKNFFQKLVQPPEQ [NH2]	0.12 ± 0.01	0.22 ± 0.03
318_AS14	[H] KIGEKLKKIGQKIANFFQKLVQPPEQ [NH2]	0.85 ± 0.10	1.50 ± 0.00
318_AS16	[H] KIGEKLKKIGQKIKNAFQKLVQPPEQ [NH2]	0.13 ± 0.03	0.23 ± 0.03
318_AS17	[H] KIGEKLKKIGQKIKNFAQKLVQPPEQ [NH2]	0.14 ± 0.01	0.28 ± 0.03
318_AS18	[H] KIGEKLKKIGQKIKNFFAKLVQPPEQ [NH2]	0.15 ± 0.00	0.20 ± 0.00
318_AS19	[H] KIGEKLKKIGQKIKNFFQALVPQPEQ [NH2]	0.18 ± 0.01	0.30 ± 0.00
318_AS20	[H] KIGEKLKKIGQKIKNFFQKAVPQPEQ [NH2]	0.23 ± 0.01	0.35 ± 0.00
318_AS21	[H] KIGEKLKKIGQKIKNFFQKLAPQPEQ [NH2]	0.12 ± 0.02	0.19 ± 0.01
318_AS22	[H] KIGEKLKKIGQKIKNFFQKLVAQPEQ [NH2]	0.12 ± 0.02	0.25 ± 0.02
318_AS23	[H] KIGEKLKKIGQKIKNFFQKLVPAPEQ [NH2]	0.12 ± 0.02	0.23 ± 0.03
318_AS24	[H] KIGEKLKKIGQKIKNFFQKLVPAEQ [NH2]	0.15 ± 0.02	0.23 ± 0.03
318_AS25	[H] KIGEKLKKIGQKIKNFFQKLVPPAQ [NH2]	0.13 ± 0.01	0.21 ± 0.01
318_AS26	[H] KIGEKLKKIGQKIKNFFQKLVQPPEA [NH2]	0.15 ± 0.00	0.25 ± 0.03

iofilm activity can be quantified by CFU determination as well as by using the metabolic dye CTB.

AS10 acts synergistically with caspofungin and amphotericin B. First, we determined the activity of AS10 against pregrown *C. albicans* biofilms. The BEC-2 was higher than 400 μM. This indicates that AS10 cannot eradicate mature *C. albicans* biofilms and hence displays antibiofilm activity against *C. albicans* only during the biofilm formation phase. Also, the OSIP108 biofilm-inhibiting peptide cannot eradicate mature biofilms. However, OSIP108 acts synergistically with commonly used antifungals such as caspofungin and amphotericin B (39). Therefore, we checked whether AS10 acts synergistically with caspofungin, am-

photericin B, and miconazole in a biofilm eradication assay. To this end, pregrown *C. albicans* biofilms were incubated with 2-fold serial dilutions of AS10 in combination with 2-fold serial dilutions of caspofungin or amphotericin B. The BEC-2 of caspofungin and amphotericin B in the presence of AS10 was determined (Table 4). In addition, FICI values were calculated for each combination by checkerboard analysis to assess potential synergy. Our results show that, although AS10 alone displays no effect on mature biofilms, different concentrations of AS10 (0.39 to 1.56 μM) act synergistically with caspofungin and amphotericin B with regard to eradication of mature biofilms (FICI ≤ 0.5; Table 4). The presence of AS10 decreased the BEC-2 of caspofungin and amphotericin B up to 8.6- and 5-fold, respectively. In contrast, AS10 does not act synergistically with miconazole (results not shown). Moreover, the synergy of AS10 with caspofungin and amphotericin B was also determined based on the more stringent BEC-5 parameter (Table 4). Similar results were obtained with these data. In the presence of AS10 (0.39 to 1.56 μM), the BEC-5 of caspofungin and amphotericin B was reduced up to 8- and 5-fold, with FICI values < 0.5. Hence, combination therapy using coadministration of AS10 and caspofungin or amphotericin B might be effective in curing biofilm-associated infections. The current repertoire of antifungal agents is limited, and some of the commonly used antifungal agents have toxic side effects. As such, combination therapy, in which the activity of existing antifungal agents is enhanced by the presence of AS10, may allow lowering of the effective dose of amphotericin B or caspofungin, thereby reducing their toxic side effects and/or economic costs (22). Furthermore, combination therapy could potentially limit the incidence of resistance to antifungal agents.

AS10 prevents biofilm formation in a mixed *C. albicans* and *S. epidermidis* biofilm. In nature, most biofilms are composed of two or more species. These mixed biofilms are clinically relevant, as nosocomial *C. albicans* bloodstream infections are often poly-

FIG 1 AS10 prevents *C. albicans* biofilm formation on titanium disks. *C. albicans* biofilms were grown in RPMI 1640 on titanium disks for 24 h in the absence (MilliQ water control) or presence of 12.5 and 6.25 μM AS10 during adhesion and biofilm formation. Afterward, biofilms were washed twice and quantified using CFU determination. The graph represents the means of the results of three biologically independent repeats ± SEM with duplicate repeats per experiment. Statistical analysis was performed using one-way ANOVA with Bonferroni's multiple-comparison test (**, *P* < 0.01; ***, *P* = 0.001). Ti, titanium.

TABLE 4 Synergistic activity of AS10 with caspofungin or amphotericin B against *C. albicans* biofilms^a

AS10 concn (μM)	Caspofungin plus AS10				Amphotericin B plus AS10				Caspofungin plus AS10				Amphotericin B plus AS10			
	BEC-2 CAS (μM) ± SEM	P value	Fold change	FICI	BEC-2 AmB (μM) ± SEM	P value	Fold change	FICI	BEC-5 CAS (μM) ± SEM	P value	Fold change	FICI	BEC-5 AmB (μM) ± SEM	P value	Fold change	FICI
0	0.65 ± 0.17	NA	NA	NA	0.67 ± 0.17	NA	NA	NA	0.70 ± 0.10	NA	NA	NA	1.23 ± 0.28	NA	NA	NA
1.56	0.08 ± 0.03	0.005	8.63	0.120	0.13 ± 0.02	0.007	5.03	0.203	0.09 ± 0.01	0.003	8.00	0.129	0.24 ± 0.03	0.004	5.14	0.171
0.78	0.09 ± 0.03	0.006	7.40	0.137	0.12 ± 0.01	0.006	5.58	0.181	0.12 ± 0.00	0.004	5.83	0.173	0.21 ± 0.04	0.003	5.97	0.158
0.39	0.15 ± 0.01	0.011	4.26	0.236	0.29 ± 0.02	0.043	2.34	0.429	0.28 ± 0.01	0.013	2.55	0.394	0.50 ± 0.03	0.021	2.48	0.245

^a AmB, amphotericin B; CAS, caspofungin; NA, not applicable. One-way ANOVA with Bonferroni's multiple-comparison test was used to compare the BEC-2 and BEC-5 of CAS and AMB in the absence of AS10 with their BEC-2 and BEC-5 in the presence of the indicated AS10 concentrations.

microbial (45) and interactions between different species in a mixed biofilm can alter their respective susceptibilities to specific antibiotics (46–48). We investigated whether AS10 can prevent biofilm formation of *C. albicans* in a mixed yeast-bacterium biofilm, i.e., one composed of *C. albicans* and *S. epidermidis*. AS10 not only reduced the number of *C. albicans* cells in the mixed biofilm but also considerably reduced the number of *S. epidermidis* cells, indicating that the biofilm-inhibitory effect of AS10 is not limited to *C. albicans* but can also be directed against bacterial biofilm formation. In a mixed biofilm, the BIC-2 for *C. albicans* is 6.25 μM, which is significantly higher than its BIC-2 in a single-species biofilm (BIC-2 = 0.22 μM) (Fig. 2). This could be due to the higher initial cell density and prolonged incubation time of the mixed biofilm protocol or due to the presence of *S. epidermidis*.

AS10 has a broad spectrum of biofilm-inhibitory activity. As AS10 inhibits biofilm development of the Gram-positive species *S. epidermidis* in a mixed biofilm, we checked whether AS10 can also inhibit biofilm formation of other bacteria, such as different Gram-negative species, including *E. coli*, *P. aeruginosa*, and *P. gingivalis*. AS10 inhibited biofilm formation of all bacterial species tested (BIC-2 = 2.6 to 20.3 μM; Table 5).

The observation that AS10 shows *C. albicans*-specific antibiofilm activity might imply that its potential clinical use is limited.

FIG 2 AS10 inhibits the formation of a mixed biofilm. Mixed biofilms of *C. albicans* and *S. epidermidis* were grown in RPMI 1640 medium in the presence or absence of AS10 during adhesion (24 h) and biofilm development (an additional 48 h). Afterward, biofilm cells were quantified by plating on YPD agar plates containing 100 mg/liter ampicillin and TSA plates containing 25 mg/liter amphotericin B to determine fungal and bacterial CFU, respectively. The percentages of *C. albicans* (filled diamonds) and *S. epidermidis* (filled circles) biofilm cells, relative to the control treatment (0.5% MilliQ water), are shown. Data represent the means and SEM of the results of three independent experiments, each consisting of two technical repeats.

However, as *C. albicans* remains the predominant fungal pathogen in humans (49–54), AS10 remains a valuable peptide in tackling *C. albicans*-associated infections. *C. albicans* is the dominant *Candida* species forming biofilms on voice prostheses (infection rate, 50% to 100%) and dentures (infection rate, 5% to 10%) (4). As AS10 can also inhibit formation of bacterial and mixed-species biofilms, this clearly broadens its application spectrum. For example, coinfection of *C. albicans* and *S. epidermidis* is a well-known problem in neonatal intensive care units and increases mortality compared to single-species infections (55, 56). The two species can form mixed biofilms on central vascular catheters, which are regularly used on these infants during the first weeks after birth and can be an important source for infection (55). Therefore, although clinical use of AS10 seems potentially limited, there are several applications where AS10 can still be valuable.

AS10 does not affect the viability and functionality of human osteoblasts, mesenchymal stromal cells, or endothelial cells. In light of its putative application as an antibiofilm coating on medical devices and implants, we assessed possible effects of AS10 on various human cell types important for implant fixation. To obtain correct integration of the implant into the bone, the coating has to be non-toxic for the surrounding cells and should not impede the functionality of the cells. Therefore, both the viability and the functionality of various human cell types, including osteoblasts, mesenchymal stromal cells, and endothelial cells, were determined in the presence of 12.5 μM AS10. AS10 did not significantly affect the viability of osteoblasts (83.7% ± 8.8% survival compared to 90.2% ± 3% [control]), mesenchymal stromal cells (87.8% ± 2.7% survival compared to 96.8% ± 2.4% [control]), and endothelial cells (99.6% ± 1.1% survival compared to 99.6% ± 0.6% [control]) after 6 days. Survival was strongly reduced by the positive-control phenol (0.05%), a known cytotoxic substance, and was 38.2% ± 11.5% for osteoblasts, 12.9% ± 15.1% for mesenchymal stromal cells, and 0% ± 0% for endothelial cells.

Furthermore, AS10 did not decrease calcium deposition of

TABLE 5 Inhibitory activity of AS10 against biofilms from different bacterial species

Bacterial species	BIC-2 ± SEM
<i>C. albicans</i> SC5314	0.22 ± 0.02
<i>C. albicans</i> DAY286	0.32 ± 0.05
<i>C. albicans</i> CAF2-1	0.67 ± 0.18
<i>E. coli</i>	2.6 ± 0.22
<i>P. aeruginosa</i>	5.4 ± 0.018
<i>P. gingivalis</i>	20.3 ± 6.42

FIG 3 AS10 does not affect tube formation of endothelial cells. Endothelial cells were cultured in M200 medium with low-serum growth supplement and treated with AS10 or 0.05% phenol. Cells were not treated (A; control) or were treated with 0.05% phenol (B) or 12.5 μ M AS10 (C). Digital images were taken 8 h after seeding.

mesenchymal stromal cells (0.179 ± 0.015 ng Ca^{2+} / μ g DNA compared to 0.167 ± 0.0045 ng Ca^{2+} / μ g DNA [control]) or osteoblasts (0.223 ± 0.008 ng Ca^{2+} / μ g DNA compared to 0.237 ± 0.020 ng Ca^{2+} / μ g DNA [control]), indicating no negative effects on the osteogenic-differentiation potential. In addition, incubation with AS10 for 8 h did not reduce the potential of endothelial cells to form tubular structures as no significant differences in the average tube lengths of endothelial cells were observed in the presence of 12.5 μ M AS10 (113 ± 17 pixels for AS10-treated endothelial cells compared to 125 ± 11 pixels for the control treatment). In contrast, treatment with 0.05% phenol significantly reduced the average tube formation after 8 h to 87 ± 8 pixels (Fig. 3). These results show that AS10 is not toxic for human osteoblasts, mesenchymal stromal cells, and endothelial cells and does not affect the functionality of these human cells, indicating that AS10 might be suitable for coating on implants to prevent biofilm formation.

Conclusions. Based on a structure-activity relationship study of the antibiofilm activity of a CRAMP-derived peptide, we identified AS10 as the most potent antibiofilm peptide which inhibits biofilm formation of *C. albicans* and various Gram-positive and Gram-negative bacteria and acts synergistically with caspofungin or amphotericin B against mature *C. albicans* biofilms. As AS10 has a broad spectrum of biofilm-inhibitory activity but does not have adverse effects on the viability or functionality of diverse human cell types involved in bone formation, AS10 is an interesting lead molecule for the development of a biofilm-preventive coating for implants.

ACKNOWLEDGMENTS

This work was supported by the European Commission's seventh Framework Programme (FP7/2007–2013) under grant agreement COATIM (Project no. 278425), the Industrial Research Fund (IOF) of the KU Leuven (knowledge platform IOF/KP/09/003), F.W.O. (Fund for Scientific Research—Flanders, W0.026.11N). and IWT Flanders (IWT/SBO/05164). K.T. and B.L. acknowledge the receipt of a postdoctoral fellowship from the Industrial Research Fund of the KU Leuven (IOFM/05/022 and IOFM/08/012, respectively). N.D. and S.R. acknowledge the receipt of a predoctoral grant of IWT Flanders (IWT101095 and IWT 083321, respectively). J.V. and H.S. are grateful for the funding received by the Strategic Basic Research of the Institute for the Promotion of Innovation through Science and Technology in Flanders under grant IWT-SBO 120050 (NEMOA), the Interdisciplinary Research Program of the Special Research Fund of the KU Leuven under grant BOF-IDO/11/008, and the F.W.O. (Fund for Scientific Research—Flanders [Belgium]) through a postdoctoral fellowship to H.S. J.M. acknowledges funding by the Inter-

university Attraction Poles Programme initiated by the Belgian Science Policy Office. The KU Leuven interfaculty MS facility was funded by the support of the Flemish government.

We thank Els Meert, Vicky De Kock, William Van Den Broeck, and Serge Beullens (CMPG, KU Leuven) for the technical assistance.

REFERENCES

- Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* 284:1318–1322. <http://dx.doi.org/10.1126/science.284.5418.1318>.
- Douglas LJ. 2003. *Candida* biofilms and their role in infection. *Trends Microbiol.* 11:30–36. [http://dx.doi.org/10.1016/S0966-842X\(02\)00002-1](http://dx.doi.org/10.1016/S0966-842X(02)00002-1).
- Kojic EM, Darouiche RO. 2004. *Candida* infections of medical devices. *Clin. Microbiol. Rev.* 17:255–267. <http://dx.doi.org/10.1128/CMR.17.2.255-267.2004>.
- Ramage G, Martinez JP, Lopez-Ribot JL. 2006. *Candida* biofilms on implanted biomaterials: a clinically significant problem. *FEMS Yeast Res.* 6:979–986. <http://dx.doi.org/10.1111/j.1567-1364.2006.00117.x>.
- Fanning S, Mitchell AP. 2012. Fungal biofilms. *PLoS Pathog.* 8:e1002585. <http://dx.doi.org/10.1371/journal.ppat.1002585>.
- Seneviratne CJ, Jin L, Samaranayake LP. 2008. Biofilm lifestyle of *Candida*: a mini review. *Oral Dis.* 14:582–590. <http://dx.doi.org/10.1111/j.1601-0825.2007.01424.x>.
- Römling U, Balsalobre C. 2012. Biofilm infections, their resilience to therapy and innovative treatment strategies. *J. Intern. Med.* 272:541–561. <http://dx.doi.org/10.1111/joim.12004>.
- Bostanci N, Belibasakis GN. 2012. *Porphyromonas gingivalis*: an invasive and evasive opportunistic oral pathogen. *FEMS Microbiol. Lett.* 333:1–9. <http://dx.doi.org/10.1111/j.1574-6968.2012.02579.x>.
- Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Hoiby N, Molin S. 2012. Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. *Nat. Rev. Microbiol.* 10:841–851. <http://dx.doi.org/10.1038/nrmicro2907>.
- Livermore DM. 2012. Current epidemiology and growing resistance of gram-negative pathogens. *Korean J. Intern. Med.* 27:128–142. <http://dx.doi.org/10.3904/kjim.2012.27.2.128>.
- Bink A, Kucharikova S, Neirincx B, Vleugels J, Van Dijk P, Cammue BP, Thevissen K. 2012. The nonsteroidal antiinflammatory drug diclofenac potentiates the *in vivo* activity of caspofungin against *Candida albicans* biofilms. *J. Infect. Dis.* 206:1790–1797. <http://dx.doi.org/10.1093/infdis/jis594>.
- Bonez PC, Dos Santos Alves CF, Dalmolin TV, Agertt VA, Mizdal CR, Flores Vda C, Marques JB, Santos RC, Anraku de Campos MM. 2013. Chlorhexidine activity against bacterial biofilms. *Am. J. Infect. Control* 41:e119–e122. <http://dx.doi.org/10.1016/j.ajic.2013.05.002>.
- Danscher G, Loch LJ. 2010. *In vivo* liberation of silver ions from metallic silver surfaces. *Histochem. Cell Biol.* 133:359–366. <http://dx.doi.org/10.1007/s00418-009-0670-5>.
- Bowdish DM, Davidson DJ, Scott MG, Hancock RE. 2005. Immunomodulatory activities of small host defense peptides. *Antimicrob. Agents Chemother.* 49:1727–1732. <http://dx.doi.org/10.1128/AAC.49.5.1727-1732.2005>.

15. Brown KL, Hancock RE. 2006. Cationic host defense (antimicrobial) peptides. *Curr. Opin. Immunol.* 18:24–30. <http://dx.doi.org/10.1016/j.coi.2005.11.004>.
16. Overhage J, Campisano A, Bains M, Torfs EC, Rehm BH, Hancock RE. 2008. Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infect. Immun.* 76:4176–4182. <http://dx.doi.org/10.1128/IAI.00318-08>.
17. Lohan S, Bisht GS. 2013. Recent approaches in design of peptidomimetics for antimicrobial drug discovery research. *Mini Rev. Med. Chem.* 13: 1073–1088. <http://dx.doi.org/10.2174/1389557511313070010>.
18. Kanthawong S, Bolscher JG, Veerman EC, van Marle J, de Soet HJ, Nazmi K, Wongratanaheewin S, Taweekaisupapong S. 2012. Antimicrobial and antibiofilm activity of LL-37 and its truncated variants against *Burkholderia pseudomallei*. *Int. J. Antimicrob. Agents* 39:39–44. <http://dx.doi.org/10.1016/j.ijantimicag.2011.09.010>.
19. Nagant C, Pitts B, Nazmi K, Vandenbranden M, Bolscher JG, Stewart PS, Dehaye JP. 2012. Identification of peptides derived from the human antimicrobial peptide LL-37 active against biofilms formed by *Pseudomonas aeruginosa* using a library of truncated fragments. *Antimicrob. Agents Chemother.* 56:5698–5708. <http://dx.doi.org/10.1128/AAC.00918-12>.
20. Wong JH, Ng TB, Legowska A, Rolka K, Hui M, Cho CH. 2011. Antifungal action of human cathelicidin fragment (LL31–37) on *Candida albicans*. *Peptides* 32:1996–2002. <http://dx.doi.org/10.1016/j.peptides.2011.08.018>.
21. Feng X, Sambanthamoorthy K, Palys T, Paravitana C. 2013. The human antimicrobial peptide LL-37 and its fragments possess both antimicrobial and antibiofilm activities against multidrug-resistant *Acinetobacter baumannii*. *Peptides* 49:131–137. <http://dx.doi.org/10.1016/j.peptides.2013.09.007>.
22. Butts A, Krysan DJ. 2012. Antifungal drug discovery: something old and something new. *PLoS Pathog.* 8:e1002870. <http://dx.doi.org/10.1371/journal.ppat.1002870>.
23. Bowdish DM, Davidson DJ, Lau YE, Lee K, Scott MG, Hancock RE. 2005. Impact of LL-37 on anti-infective immunity. *J. Leukoc. Biol.* 77: 451–459. <http://dx.doi.org/10.1189/jlb.0704380>.
24. Hell E, Giske CG, Nelson A, Romling U, Marchini G. 2010. Human cathelicidin peptide LL37 inhibits both attachment capability and biofilm formation of *Staphylococcus epidermidis*. *Lett. Appl. Microbiol.* 50:211–215. <http://dx.doi.org/10.1111/j.1472-765X.2009.02778.x>.
25. Kanthawong S, Nazmi K, Wongratanaheewin S, Bolscher JG, Wuthiekanun V, Taweekaisupapong S. 2009. *In vitro* susceptibility of *Burkholderia pseudomallei* to antimicrobial peptides. *Int. J. Antimicrob. Agents* 34:309–314. <http://dx.doi.org/10.1016/j.ijantimicag.2009.05.012>.
26. Tsai PW, Yang CY, Chang HT, Lan CY. 2011. Human antimicrobial peptide LL-37 inhibits adhesion of *Candida albicans* by interacting with yeast cell-wall carbohydrates. *PLoS One* 6:e17755. <http://dx.doi.org/10.1371/journal.pone.0017755>.
27. Chang HT, Tsai PW, Huang HH, Liu YS, Chien TS, Lan CY. 2012. LL37 and hBD-3 elevate the beta-1,3-exoglucanase activity of *Candida albicans* Xog1p, resulting in reduced fungal adhesion to plastic. *Biochem. J.* 441: 963–970. <http://dx.doi.org/10.1042/BJ20111454>.
28. Fonzi WA, Irwin MY. 1993. Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* 134:717–728.
29. Plaine A, Walker L, Da Costa G, Mora-Montes HM, McKinnon A, Gow NA, Gaillardin C, Munro CA, Richard ML. 2008. Functional analysis of *Candida albicans* GPI-anchored proteins: roles in cell wall integrity and caspofungin sensitivity. *Fungal Genet. Biol.* 45:1404–1414. <http://dx.doi.org/10.1016/j.fgb.2008.08.003>.
30. Kaur R, Ma B, Cormack BP. 2007. A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. *Proc. Natl. Acad. Sci. U. S. A.* 104:7628–7633. <http://dx.doi.org/10.1073/pnas.0611195104>.
31. Sullivan DJ, Westerneng TJ, Haynes KA, Bennett DE, Coleman DC. 1995. *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. *Microbiology* 141(Pt 7):1507–1521. <http://dx.doi.org/10.1099/13500872-141-7-1507>.
32. Thevissen K, Pellens K, De Brucker K, Francois IE, Chow KK, Meert EM, Meert W, Van Minnebruggen G, Borgers M, Vroome V, Levin J, De Vos D, Maes L, Cos P, Cammue BP. 2011. Novel fungicidal benzyl-sulfanyl-phenylguanidines. *Bioorg. Med. Chem. Lett.* 21:3686–3692. <http://dx.doi.org/10.1016/j.bmcl.2011.04.075>.
33. Sambrook JF, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
34. Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, Villanueva J, Wei T, Ausubel FM. 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc. Natl. Acad. Sci. U. S. A.* 103:2833–2838. <http://dx.doi.org/10.1073/pnas.0511100103>.
35. Coykendall AL, Kaczmarek FS, Slots J. 1980. Genetic-heterogeneity in bacteroides-*asaccharolyticus* (holdeman and moore 1970) finegold and barnes 1977 (approved lists, 1980) and proposal of bacteroides-*gingivalis* sp-nov and bacteroides-*macacae* (slots and genco) comb-nov. *Int. J. Syst. Bacteriol.* 30:559–564. <http://dx.doi.org/10.1099/00207713-30-3-559>.
36. O'Brien J, Wilson I, Orton T, Pognan F. 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem.* 267:5421–5426. <http://dx.doi.org/10.1046/j.1432-1327.2000.01606.x>.
37. Tellier R, Krajden M, Grigoriev GA, Campbell I. 1992. Innovative endpoint determination system for antifungal susceptibility testing of yeasts. *Antimicrob. Agents Chemother.* 36:1619–1625. <http://dx.doi.org/10.1128/AAC.36.8.1619>.
38. Delattin N, De Brucker K, Vandamme K, Meert E, Marchand A, Chaltin P, Cammue BP, Thevissen K. 2014. Repurposing as a means to increase the activity of amphotericin B and caspofungin against *Candida albicans* biofilms. *J. Antimicrob. Chemother.* 69:1035–1044. <http://dx.doi.org/10.1093/jac/dkt449>.
39. Delattin N, De Brucker K, Craik DJ, Cheneval O, Frohlich M, Veber M, Girandon L, Davis TR, Weeks AE, Kumamoto CA, Cos P, Coenye T, De Coninck B, Cammue BP, Thevissen K. 2014. Plant-derived decapeptide OSIP108 interferes with *Candida albicans* biofilm formation without affecting cell viability. *Antimicrob. Agents Chemother.* 58:2647–2656. <http://dx.doi.org/10.1128/AAC.01274-13>.
40. Janssens JC, Steenackers H, Robijns S, Gellens E, Levin J, Zhao H, Hermans K, De Coster D, Verhoeven TL, Marchal K, Vanderleyden J, De Vos DE, De Keersmaecker SC. 2008. Brominated furanones inhibit biofilm formation by *Salmonella enterica* serovar Typhimurium. *Appl. Environ. Microbiol.* 74:6639–6648. <http://dx.doi.org/10.1128/AEM.01262-08>.
41. Steenackers HP, Ermolat'ev DS, Savaliya B, Weerd AD, Coster DD, Shah A, Van der Eycken EV, De Vos DE, Vanderleyden J, De Keersmaecker SC. 2011. Structure-activity relationship of 2-hydroxy-2-aryl-2,3-dihydro-imidazo[1,2-a]pyrimidinium salts and 2N-substituted 4(5)-aryl-2-amino-1H-imidazoles as inhibitors of biofilm formation by *Salmonella Typhimurium* and *Pseudomonas aeruginosa*. *Bioorg. Med. Chem.* 19:3462–3473. <http://dx.doi.org/10.1016/j.bmc.2011.04.026>.
42. Odds FC. 2003. Synergy, antagonism, and what the checkerboard puts between them. *J. Antimicrob. Chemother.* 52:1. <http://dx.doi.org/10.1093/jac/dkg301>.
43. Boonen K, Baggerman G, D'Hertog W, Husson SJ, Overbergh L, Mathieu C, Schoofs L. 2007. Neuropeptides of the islets of Langerhans: a peptidomics study. *Gen. Comp. Endocrinol.* 152:231–241. <http://dx.doi.org/10.1016/j.ygcen.2007.05.002>.
44. Shin SY, Kang SW, Lee DG, Eom SH, Song WK, Kim JI. 2000. CRAMP analogues having potent antibiotic activity against bacterial, fungal, and tumor cells without hemolytic activity. *Biochem. Biophys. Res. Commun.* 275:904–909. <http://dx.doi.org/10.1006/bbrc.2000.3269>.
45. Harriott MM, Noverr MC. 2011. Importance of *Candida*-bacterial polymicrobial biofilms in disease. *Trends Microbiol.* 19:557–563. <http://dx.doi.org/10.1016/j.tim.2011.07.004>.
46. Harriott MM, Noverr MC. 2009. *Candida albicans* and *Staphylococcus aureus* form polymicrobial biofilms: effects on antimicrobial resistance. *Antimicrob. Agents Chemother.* 53:3914–3922. <http://dx.doi.org/10.1128/AAC.00657-09>.
47. Harriott MM, Noverr MC. 2010. Ability of *Candida albicans* mutants to induce *Staphylococcus aureus* vancomycin resistance during polymicrobial biofilm formation. *Antimicrob. Agents Chemother.* 54:3746–3755. <http://dx.doi.org/10.1128/AAC.00573-10>.
48. Adam B, Baillie GS, Douglas LJ. 2002. Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. *J. Med. Microbiol.* 51:344–349.
49. Marr KA. 2004. Invasive *Candida* infections: the changing epidemiology. *Oncology (Williston Park)* 18(Suppl 13):9–14.
50. Pfaller MA, Diekema DJ. 2010. Epidemiology of invasive mycoses in North America. *Crit. Rev. Microbiol.* 36:1–53. <http://dx.doi.org/10.3109/104008410903241444>.

51. Pfaller MA, Messer SA, Moet GJ, Jones RN, Castanheira M. 2011. *Candida* bloodstream infections: comparison of species distribution and resistance to echinocandin and azole antifungal agents in Intensive Care Unit (ICU) and non-ICU settings in the SENTRY Antimicrobial Surveillance Program (2008-2009). *Int. J. Antimicrob. Agents* 38:65–69. <http://dx.doi.org/10.1016/j.ijantimicag.2011.02.016>.
52. Pfaller M, Neofytos D, Diekema D, Azie N, Meier-Kriesche HU, Quan SP, Horn D. 2012. Epidemiology and outcomes of candidemia in 3648 patients: data from the Prospective Antifungal Therapy (PATH Alliance(R)) registry, 2004–2008. *Diagn. Microbiol. Infect. Dis.* 74:323–331. <http://dx.doi.org/10.1016/j.diagmicrobio.2012.10.003>.
53. Diekema D, Arbefeville S, Boyken L, Kroeger J, Pfaller M. 2012. The changing epidemiology of healthcare-associated candidemia over three decades. *Diagn. Microbiol. Infect. Dis.* 73:45–48. <http://dx.doi.org/10.1016/j.diagmicrobio.2012.02.001>.
54. Wisplinghoff H, Ebberts J, Geurtz L, Stefanik D, Major Y, Edmond MB, Wenzel RP, Seifert H. 2014. Nosocomial bloodstream infections due to *Candida* spp. in the U. S. A.: species distribution, clinical features and antifungal susceptibilities. *Int. J. Antimicrob. Agents* 43:78–81.
55. Kaufman DA, Brown AT, Eisenhuth KK, Yue J, Grossman LB, Hazen KC. 2014. More serious infectious morbidity and mortality associated with simultaneous candidemia and coagulase-negative staphylococcal bacteremia in neonates and *in vitro* adherence studies between *Candida albicans* and *Staphylococcus epidermidis*. *Early Hum. Dev.* 90(Suppl 1): S66–S70. [http://dx.doi.org/10.1016/S0378-3782\(14\)70021-0](http://dx.doi.org/10.1016/S0378-3782(14)70021-0).
56. Venkatesh MP, Pham D, Fein M, Kong L, Weisman LE. 2007. Neonatal coinfection model of coagulase-negative *Staphylococcus* (*Staphylococcus epidermidis*) and *Candida albicans*: fluconazole prophylaxis enhances survival and growth. *Antimicrob. Agents Chemother.* 51:1240–1245. <http://dx.doi.org/10.1128/AAC.01298-06>.