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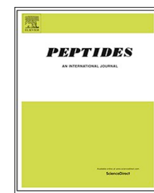
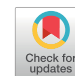


Structure-activity relationship study of the antimicrobial CRAMP-derived peptide CRAMP20-33



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ABSTRACT

We report here on the structure-activity relationship study of a 14 amino acid fragment of the cathelicidin-related antimicrobial peptide (CRAMP), CRAMP20-33 (KKIGQKIKNFFQKL). It showed activity against *Escherichia coli* and filamentous fungi with IC_{50} values below 30 μM and 10 μM, respectively. CRAMP20-33 variants with glycine at position 23 substituted by phenylalanine, leucine or tryptophan showed 2- to 4-fold improved activity against *E. coli* but not against filamentous fungi. Furthermore, the most active single-substituted peptide, CRAMP20-33 G23 W (IC_{50} = 2.3 μM against *E. coli*), showed broad-spectrum activity against *Candida albicans, Staphylococcus epidermidis* and *Salmonella* Typhimurium. Introduction of additional arginine substitutions in CRAMP20-33 G23 W, more specifically in CRAMP20-33 G23 W N28R or CRAMP20-33 G23 W Q31R, resulted in 3-fold increased activity against *S. epidermidis* (IC_{50} = 4 μM and 4.8 μM, respectively) as compared to CRAMP20-33 G23 W (IC_{50} = 15.1 μM) but not against the other pathogens tested. In general, double-substituted variants were non-toxic for human HepG2 cells, pointing to their therapeutic potential.

1. Introduction

The increase and spread of antibiotic resistance necessitates the search for new sources of potent, broad-spectrum antimicrobials. Interesting candidates in this context are antimicrobial peptides (AMPs), which are major components of the innate immunity and host defense of many plants and animals [1]. In this study we focus on the mouse cathelicidin-related antimicrobial peptide (CRAMP), which is the homologue of the human LL-37 [2,44] (Table 1). CRAMP is a cationic antimicrobial host defense peptide, exhibiting low propensity for resistance development [3]. It displays potent antimicrobial activity against both Gram-negative and Gram-positive bacteria and against the human fungal pathogen *Candida albicans* [4–6]. Earlier reports demonstrated the sequence of CRAMP starting from amino acid 16 till 33 (CRAMP16-33; Table 1) to be mainly responsible for the antibacterial activity of CRAMP [4] through inhibition of bacterial cytokinesis [7].

We previously identified another CRAMP variant, starting from amino acid 18 till 35 (CRAMP18-35 G23 A; Table 1), which displays potent antibiofilm activity against *Escherichia coli, Pseudomonas aeruginosa*, *Porphyromonas gingivalis*, *Staphylococcus epidermidis* and *C. albicans* [3]. Based on the amino acid sequences of all CRAMP-variants with documented antimicrobial activity [3], we identified a minimal core sequence of CRAMP from amino acid 20 to 33 (CRAMP20-33; Table 1). This CRAMP20-33 peptide has 6 out of the 14 amino acids identical to those of a modified truncated LL-37 antimicrobial peptide, P60.4. These modifications include amino acid substitutions, acetylations and amidations to enhance the peptide's amphipaticity and stability (Table 1). Interestingly, it was reported earlier that P60.4 has improved activity against *E. coli, P. aeruginosa* and *C. albicans* compared to the native LL-37 [8–10].

In this study, we assessed the antimicrobial activity of CRAMP20-33 against a broad panel of microbial pathogens, including the bacteria *E. coli*, *Salmonella* Typhimurium and *S. epidermidis*, the filamentous fungi *Fusarium oxysporum* and *Aspergillus fumigatus* and the yeast species *C. albicans* and *C. glabrata*. Based on its antimicrobial activity against filamentous fungi and *E. coli*, we performed a structure-activity relationship study of CRAMP20-33 using a whole amino acid scan. Next, CRAMP20-33 double-substituted variants combining the most promising single substitution with arginine-substitutions were synthetized and their antimicrobial activity was determined in order to identify CRAMP20-33 variants with further improvements in their antimicrobial activity.

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Table 1
Amino acid sequences of native LL-37 and CRAMP, and their derivatives. Conserved residues are represented in bold.

PEPTIDES	AMINO ACID SEQUENCES	REFERENCES
LL-37	LLGDFFRKSKEK**IGKEFKRIVQRIKDFLRNLVPRTE**S	[44]
P60.4	**IGKEFKRIV**E**RIK**R**FLR**E**LV**RPLR	[8]
CRAMP	ISRLAGLLRKGGEKIGEKL**KKI**G**QKIKNFFQKL**VPQPE	[2]
CRAMP16-33	GEKL**KKI**G**QKIKNFFQKL**	[4]
CRAMP18-35	KL**KKI**A**QKIKNFFQKL**VP	[3]
CRAMP20-33	**KKI**G**QKIKNFFQKL**	This study

2. Materials and methods

2.1. Strains and chemicals

Strains *C. albicans* SC5314 [11], *C. glabrata* BG2 [12], *F. oxysporum* Fo5176 [13], *A. fumigatus* CBS117202, *E. coli* K12 MG1655 [14], *E. coli* UTI89 [15], *S. enterica* serovar Typhimurium ATCC/SL 14028 (*S.* Typhimurium) and *S. epidermidis* [16] were used in this study. Overnight cultures of *C. albicans* and *C. glabrata* were grown in YPD (1% yeast extract, 2% peptone and 2% dextrose, Difco) at 30 °C. Overnight cultures of *E. coli* and *S.* Typhimurium were grown in LB (L-broth, LAB) at 37 °C. Overnight culture of *S. epidermidis* was grown in TSB (30 g full strength Trypticase soy broth in 1 L water (3%), BBL) at 37 °C. *F. oxysporum* and *A. fumigatus* were grown in half strength PDB (1.2% potato dextrose broth, Difco). RPMI-1640 medium (Roswell Park Memorial Institute-1640 medium) with L-glutamine and without sodium bicarbonate (Sigma, St Louis, USA) was buffered with MOPS (morpholinepropanesulfonic acid, Sigma, St Louis, USA) to pH 7. Cell culture MEM medium (minimal essential medium, Thermo-Fisher, USA). All CRAMP-derivatives peptides were pure (> 95% purity), except those used in the structure-activity relationship study (Fig. 1) which were of crude purity. All peptides were produced by Solid phase peptide synthesis (SPPS) using standard Fmoc/tBu protocols at Pepscan (Leystad, The Netherlands). Furthermore, they were purified using reversed phase HPLC and lyophilized. Purity and identity of the peptides were assayed by analytical UPLC/MS. Finally, the peptides were stored at −20 °C diluted in MQ to a 2 mM stock concentration.

2.2. Antimicrobial activity assay

The minimal concentration of CRAMP20-33 and its variants resulting in 50% growth inhibition (IC_{50}) of various pathogenic bacteria, yeasts and fungi [17] was determined. In addition, the minimal concentration resulting in 100% growth inhibition (IC_{100}) was determined in the antibacterial assays. To this end, the standard Clinical and Laboratory Standards Institute (CLSI) protocols M38-A for fungi, M27-A2 for yeast or M07-A9 for bacteria were followed with minor modifications [18–20]: two-fold dilution series of the peptides were prepared in MQ. Then, 10 µl of these series were added in 96-well microtiter plates to each (i) 90 µL ½ PDB containing 5×10^4 spores/ml (*F. oxysporum* or *A. fumigatus*), (ii) 90 µl TSB containing approximately 10^5 cells/ml (*E. coli, S. epidermidis* or *S.* Typhimurium) diluted from overnight cultures, (iii) and to 90 µl RPMI-1640 containing approximately 10^3 cells/ml (*C. albicans* or *C. glabrata*) diluted from overnight cultures. PDB and TSB are media that are routinely used for testing cationic peptides [21,22]. Fungi were incubated for 72 h at 25 °C, whereas yeast and bacterial species were incubated for 24 h at 37 °C. The plates were covered with EASYseal™ sealing film (Greiner Bio-One) during incubation period to avoid evaporation. MQ water was used as a negative control. Growth of the microorganisms was determined using a multireader (absorbance 490 nm).

2.3. Human cell viability assay

The cytotoxicity of native CRAMP20-33 and its variants was tested against the hepatic cell line HepG2. In a 96-well plate, 10 000 HepG2 cells per well were plated in MEM medium. After 24 h, cells were incubated with the different CRAMP20-33 variants. After another 24 h, the peptides were replaced with media containing the cell viability dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, St Louis, USA). The cells were incubated with the MTT reagent for 2 h and replaced with 50 µL DMSO (dimethyl sulfoxide, Sigma, St Louis, USA). The MTT signal, indicative of cell viability, was measured by absorbance at 560 nm.

2.4. Statistical analysis

Data were analyzed using GraphPad Prism version 6.01 (GraphPad Software, Inc., CA, USA). For dose-response data, nonlinear regression (curve fit) was used to generate sigmoidal curves. The concentration required to cause 50% growth inhibition (IC_{50}) was derived from the whole dose-response curves.

3. Results

3.1. CRAMP20-33 is active against E. coli and filamentous fungi

Antimicrobial activity assays with CRAMP20-33 were performed against several bacteria, fungi and yeasts (Table 2). The results revealed no substantial activity of CRAMP20-33 against the two *Candida* species, *S.* Typhimurium and *S. epidermidis*, even at the highest concentration tested ($IC_{50} > 50$ µM). However, CRAMP20-33 demonstrated antimicrobial activity against all filamentous fungi tested and *E. coli* with IC_{50} values below 10 µM and 30 µM, respectively. Next, we performed a whole amino acid scan of CRAMP20-33 to identify variants with further improved antimicrobial activity (decreased IC_{50}-value) against *E. coli*, filamentous fungi and yeasts as compared to the native CRAMP20-33.

3.2. Structure-activity study of CRAMP20-33

The structure-activity relationship study of CRAMP20-33 resulted in a panel of 266 CRAMP20-33 variants in which each amino acid of CRAMP20-33 was individually replaced with all 19 other common amino acids. The IC_{50} of all CRAMP20-33 variants was determined from dose-response curves and the fold change (FC) in the IC_{50} of these variants relative to that of native CRAMP20-33 was calculated. The data of CRAMP20-33 variants activity is represented as heat maps based on the IC_{50} values (Fig. 1). These heat maps indicate that CRAMP20-33's activity could be improved against *E. coli* but not against filamentous fungi. Most notably, the amino acids of CRAMP20-33 at positions 26, 29, 30 and 33 appeared of high importance for its antifungal activity as substitution of these amino acids by almost any other amino acid resulted in more than 4-fold reduced activity (Fig. 1b-c).

From the heat map of CRAMP20-33's activity against *E. coli*

(a) *Escherichia coli*

CRAMP 20-33													
K20	K21	I22	G23	Q24	K25	I26	K27	N28	F29	F30	Q31	K32	L33
K	K	I	G	Q	K	I	K	N	F	F	Q	K	L
A	A	A	D	A	A	A	A	D	A	A	D	A	A
C	C	C	E	C	C	C	C	E	C	C	E	C	C
D	D	D	H	D	D	D	D	G	D	D	H	D	D
E	E	E	K	E	E	E	E	H	E	E	I	E	E
F	F	F	N	F	G	F	F	I	G	G	P	F	F
G	G	G	P	G	H	G	G	L	H	H	A	G	G
H	H	H	Q	H	N	H	H	M	I	I	C	H	H
I	I	K	S	I	P	K	I	P	K	K	F	I	I
L	L	M	T	K	Q	L	L	S	L	M	G	L	K
M	M	N	A	L	S	M	M	T	M	N	K	M	M
P	N	P	C	M	T	N	N	V	N	P	L	N	N
Q	P	Q	I	N	V	P	P	Y	P	Q	M	P	P
R	Q	R	M	P	F	Q	Q	A	Q	R	N	Q	Q
S	S	S	R	R	I	R	S	C	R	S	R	R	R
T	T	T	V	S	L	S	T	F	S	T	S	S	S
V	V	V	Y	T	M	T	V	K	T	V	T	T	T
W	Y	Y	F	V	R	V	W	Q	V	W	V	V	V
Y	R	L	L	W	W	W	Y	R	Y	Y	W	Y	W
N	W	W	W	Y	Y	Y	R	W	W	L	Y	W	Y

(b) *Fusarium oxysporum*

CRAMP 20-33													
K20	K21	I22	G23	Q24	K25	I26	K27	N28	F29	F30	Q31	K32	L33
K	K	I	G	Q	K	I	K	N	F	F	Q	K	L
D	D	D	D	Y	D	D	D	P	D	D	P	I	D
E	E	E	E	A	P	E	P	E	E	E	F	P	E
C	I	N	A	C	E	N	E	F	G	G	I	D	G
Q	N	A	C	D	C	P	N	I	N	N	A	F	V
A	Q	C	F	E	G	Q	C	A	P	P	C	L	C
F	V	G	H	F	N	G	F	C	Q	Q	D	M	I
G	Y	H	I	G	Q	H	G	D	S	R	E	V	M
H	A	K	K	H	A	R	Q	G	H	H	G	W	N
I	C	P	L	I	F	S	S	H	T	K	H	A	P
L	F	Q	M	K	H	T	T	K	K	S	K	C	S
M	G	R	N	L	I	C	V	L	R	T	L	E	T
N	H	S	P	M	L	K	A	M	A	A	M	G	W
P	L	T	Q	N	M	Y	H	Q	C	C	N	N	Y
R	M	F	R	P	R	A	I	R	I	M	R	Q	A
S	P	L	S	R	S	M	L	S	L	V	S	S	F
T	R	M	T	S	T	W	M	T	M	Y	T	T	H
V	S	V	V	T	V	F	R	V	V	I	V	Y	K
W	T	W	W	V	W	L	W	W	Y	L	W	H	Q
Y	W	Y	Y	W	Y	V	Y	Y	W	W	Y	R	R

(c) *Aspergillus fumigatus*

CRAMP 20-33													
K20	K21	I22	G23	Q24	K25	I26	K27	N28	F29	F30	Q31	K32	L33
K	K	I	G	Q	K	I	K	N	F	F	Q	K	L
A	C	A	C	C	C	A	D	E	A	A	P	A	A
C	D	C	D	D	D	C	E	P	C	C	D	D	C
D	E	D	E	E	E	D	G	T	D	D	E	E	D
E	I	E	F	I	G	E	N	D	E	E	I	N	E
P	L	G	P	P	N	G	P	G	G	G	Y	P	F
Q	M	K	N	F	P	H	Q	I	H	H	A	Q	G
N	P	N	Q	L	Q	K	S	S	K	K	C	C	H
R	Q	P	S	M	A	N	T	V	L	N	F	F	I
S	V	Q	T	N	S	P	A	A	M	P	G	G	K
T	A	S	V	V	T	Q	C	C	N	Q	H	I	N
V	F	F	A	W	F	R	F	F	P	R	K	L	P
Y	G	H	H	Y	H	S	I	H	Q	S	L	M	Q
F	N	M	I	A	I	T	V	K	R	T	M	S	S
G	S	R	K	G	L	Y	Y	L	S	V	N	T	T
H	T	T	L	H	M	M	H	M	T	Y	R	V	V
I	W	V	M	K	R	W	L	Q	V	I	S	W	W
L	Y	L	R	R	V	F	M	R	Y	L	T	Y	Y
M	H	W	W	S	W	L	R	W	I	M	V	H	M
W	R	Y	Y	T	Y	V	W	Y	W	W	W	R	R

Increased FC in IC50 (reduced activity)

Decreased FC in IC50 (increased activity)

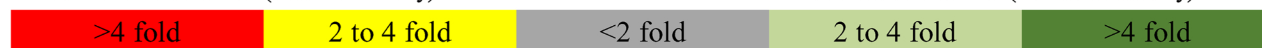

Fig. 1. Structure-activity relationship study of CRAMP20-33′s activity against (a) *E. coli*, (b) *F. oxysporum* and (c) *A. fumigatus*. Black: native CRAMP20-33 sequence. Antimicrobial activity of 266 CRAMP20-33 variants in which every amino acid was replaced by any of the other amino acids was determined against *E.coli* and filamentous fungi in at least two biologically independent experiments. From dose-response curves, IC_{50} values were determined for all variants. Variants are vertically ranked from lowest to highest antimicrobial activity. Variants with the same score are ranked alphabetically. Colors indicate increased or decreased average fold changes (FC) in IC_{50} of the variants relative to native CRAMP20-33.

(Fig. 1a), it is clear that most substitutions reduced its antibacterial potential by 2- to 4-fold. The introduction of proline (P) or histidine (H), or of negatively charged amino acids like aspartic acid (D) or glutamic acid (E) at any position, as well as the introduction of glutamine (Q) at any position except for position 28, resulted in decreased antibacterial activity. Position 31 allowed most amino acid substitutions without affecting the activity against *E. coli*, followed by positions 23, 25 and 28. Only a few CRAMP20-33 variants in which the glycine (G) at position 23 was replaced by amino acids with a hydrophobic side chain like phenylalanine (F), leucine (L) or tryptophan (W), were characterized by 2- to 4-fold improved antibacterial activity compared to CRAMP20-33. However, note that the antibacterial activity of CRAMP20-33 was not increased when F, W or L were introduced at other positions than at position 23. Furthermore, introduction of arginine (R) or lysine (K) also failed to improve the antibacterial activity of CRAMP20-33. Note that substitution of isoleucine (I) with L was possible at position 22 without affecting the activity of CRAMP20-33. In contrast, substitutions of I with L or L with I at positions 26 and 33, respectively, resulted in more than 2-fold decreased activity. Hence, in general, it seems that R substitutions are possible at all cationic positions without affecting the activity of CRAMP20-33 while I or L substitutions are only possible at some hydrophobic positions.

Table 2
Antimicrobial activity of native CRAMP20-33 and its single- and double-substituted variants against bacterial, yeast and fungal pathogens. IC_{50} values (μM) are represented as means of at least two biologically independent experiments. IC_{100} values (μM) were included for bacterial pathogens.

Peptides	IC_{50} (μM) *(IC_{100} (μM))*						
	C. albicans	*C. glabrata*	*F. oxysporum*	*A. fumigatus*	*E. coli* K12	*E. coli* UTI89	*S. epidermidis*	*S.* Typhimurium
CRAMP20-33	> 50	> 50	1.7	5.3	28.1 *(50)*	> 50 *(> 50)*	> 50 *(> 50)*	> 50 *(> 50)*
CRAMP20-33 G23W	23.9	> 50	3.7	19.4	2.3 *(6.25)*	1.5 *(6.25)*	15.1 *(29)*	23.3 *(50)*
CRAMP20-33 G23 W K21R	21.6	> 50	5.9	19.5	2.9 *(6.25)*	2.0 *(6.25)*	11.0 *(25)*	15.8 *(25)*
CRAMP20-33 G23 W K25R	25.6	> 50	4.3	18.8	3.5 *(7)*	1.9 *(6.25)*	11.8 *(50)*	12.3 *(29)*
CRAMP20-33 G23 W K27R	> 50	> 50	15.7	34.1	8.3 *(12.5)*	5.2 *(12.5)*	> 50 *(> 50)*	> 50 *(> 50)*
CRAMP20-33 G23 W N28R	22.9	> 50	7.2	15.9	> 50 *(> 50)*	> 50 *(> 50)*	4.0 *(7.5)*	17.4 *(50)*
CRAMP20-33 G23 W Q31R	24.4	> 50	6.9	13.2	10.6 *(50)*	6.6 *(25)*	4.8 *(12.5)*	26.8 *(> 50)*

3.3. CRAMP20-33 G23 W has improved antimicrobial activity

We assessed the antimicrobial activity of the best single-substituted variant, CRAMP20-33 G23 W, against the different microbial pathogens mentioned in Section 2.1 (Table 2). CRAMP20-33 G23 W showed increased activity against the Gram-negative bacterial pathogen (*S.* Typhimurium) and the Gram-positive bacterial pathogen (*S. epidermidis*) as compared to native CRAMP20-33. Furthermore, this CRAMP20-33 variant showed increased activity against the yeast *C. albicans*. However, CRAMP20-33's activity could not be improved by the introduction of the single-substitution G23 W against the yeast *C. glabrata*.

3.4. Improved double-substituted variants of CRAMP20-33

Next we designed CRAMP20-33 double-substituted variants. To this end, arginine (R) was additionally introduced in the sequence of CRAMP20-33 G23 W at all the positions that allow these substitutions without affecting the activity of CRAMP20-33 against *E. coli* as documented in Fig. 1a. Arginine (R) substitutions are known to improve the antimicrobial activity of a peptide to a greater extent than substitutions by other positively charged amino acids. This is probably due to the superior capacity of R to form H-bonds, favoring interfacial binding and producing more dramatic perturbations of the membrane [23]. We tested the antimicrobial activity of the resulting double-substituted variants of CRAMP20-33, more specifically G23 W K21R, G23 W K25R, G23 W K27R, G23 W N28R and G23 W Q31R, against the panel of pathogenic bacteria and fungi.

In general, the CRAMP20-33 G23 W could not be further improved by the introduction of additional R substitutions regarding its activity against *E.coli, S.* Typhimurium nor *C. albicans.* However, the double-substituted CRAMP20-33 variants, CRAMP20-33 G23 W N28R and CRAMP20-33 G23 W Q31R, showed increased activity (IC_{50} < 5 μM; IC_{100} < 15 μM) against *S. epidermidis* as compared to the single-substituted CRAMP20-33 G23 W (IC_{50} = 15.1 μM; IC_{100} = 29 μM). These data indicate that the introduction of a second substitution in CRAMP20-33 G23 W can result in a further improvement of CRAMP20-33's activity against *S. epidermidis.* No further improvements in CRAMP20-33's activity against filamentous fungi (*F. oxysporum* and *A. fumigatus*) could be obtained. Moreover, CRAMP20-33 remained inactive against *C. glabrata* even when double-substitutions were introduced.

Finally, we assessed the toxicity of native CRAMP20-33 and its variants against human HepG2 cells (Fig. 2). In general, these peptides did not affect the viability and functionality of these cells at concentrations relevant for their antimicrobial activity. The half maximal effective concentration (EC50) of native CRAMP20-33, CRAMP20-33 G23 W and double-substituted variants of CRAMP20-33, including G23 W K21R, G23 W K27R, G23 W N28R and G23 W Q31R, was higher than the maximal concentration tested (50 μM). Thus these variants do not exert toxicity against human cells at concentrations relevant for their selective activity, such as exemplified for CRAMP20-33 G23 W K21R against *E. coli* (IC_{50} ≤ 2.9 μM; IC_{100} = 6.25 μM), *C. albicans* (IC_{50} = 21.6 μM), *S. epidermidis* (IC_{50} = 11 μM; IC_{100} = 25 μM) and *S.* Typhimurium (IC_{50} = 15.8 μM; IC_{100} = 25 μM). Only for one of the double-substituted variants, CRAMP20-33 G23 W K25R, the EC50 against human HepG2 cells was 50 μM, but still at least 2-fold higher than its IC_{50} values against the different pathogens.

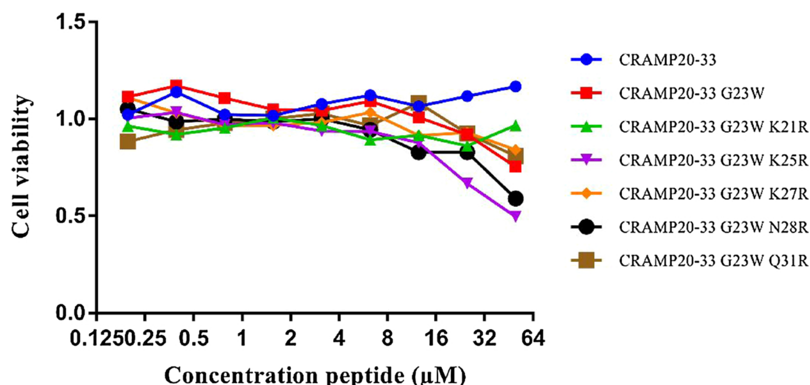

Fig. 2. Cytotoxicity of native CRAMP20-33 and its variants against human HepG2 cells. Data represent cell viability relative to untreated control from three biologically independent experiments, each one from three technical replicates.

4. Discussion

AMPs are oligopeptides that form part of the innate immune system of most living organisms and that have a broad spectrum of activity against bacteria and fungi [24,25]. Here, we determined the antimicrobial activity of a 14 amino acid fragment of CRAMP, being CRAMP20-33, and of 266 of its variants, against several pathogenic bacteria and fungi. We demonstrated that the activity of CRAMP20-33 can be improved against *E. coli* by substitutions of the G at position 23 for F, L or W. Glycine is the only amino acid without a side chain, thereby allowing flexibility of the peptide [26]. This indicates that a more constrained tertiary structure of CRAMP20-33 was generated by the introduction of F, W or L which increased the hydrophobicity of the peptide thereby possibly improving its antibacterial activity. Moreover, it has been demonstrated before that increasing the hydrophobicity of an antimicrobial peptide in general improves its antimicrobial activity due to a better interaction of the peptide with the membrane surface of cells [27]. Furthermore, though introduction of positively charged amino acids, like R or K is also reported to improve antimicrobial activity of peptides [28,29], neither of these substitutions improved CRAMP20-33′s antibacterial activity. The reason why R or K substitutions did not improve the activity of CRAMP20-33 against *E. coli* may be because the charge density along the peptide becomes too high to allow it to efficiently adopt an active structure [30,31]. In addition, the introduction of additional R substitutions to CRAMP20-33 G23 W did not further improve the activity of CRAMP20-33 against *E. coli.* However, the double-substituted CRAMP20-33 G23 W N28R and CRAMP20-33 G23 W Q31R exhibited improved activity against *S. epidermidis* as compared to CRAMP20-33 G23 W. Interestingly, the resulting peptides were non-toxic for human HepG2 cells at concentrations relevant for their antimicrobial activity. Moreover, the single- and double-substituted variants of CRAMP20-33 were active against *S.* Typhymurium and *C. albicans* but not against filamentous fungi nor *C. glabrata.*

AMPs may be amongst the most promising potential antibiotics, due to advantages such as high antimicrobial activity and low propensity for resistance development [32–34]. They can be used as AMP-based antibiotic formulations or in combination with other antimicrobial compounds such as ‘conventional’ antibiotics thereby enhancing the activity of the antibiotics [25,34–36]. Currently, various AMP-based antibiotic formulations are on the market such as daptomycin, vancomycin, bacitracin and colistin for treating bacterial infections [24,25]. The latter, however, displays general toxicity [37] and resistance occurrence has been reported [38]. Interestingly, it has been described that conjugates of vancomycin and CRAMP have broad-spectrum activity, in contrast to CRAMP and vancomycin alone. Moreover, these conjugates have improved antibacterial and antibiofilm activities as compared to mixtures of CRAMP and vancomycin [1]. In addition, there are a number of peptide-based drugs that are currently undergoing clinical phase II and III testing. Examples are omiganan (MBI 226), which is a peptide derivative from the AMP indolicidin, and LL-37, the human homologue of CRAMP [39–41]. LL-37 successfully overcame phase I/II of the clinical study (trial registration: EU Clinical Trials Register 2012-002100-41) as a potent candidate to treat venous leg ulcers [42,45]. LL-37 seems to be highly effective for the topical treatment of polymicrobially infected wounds [39]. Furthermore, as CRAMP20-33 variants are similar to LL-37 regarding their primary structure [43], they show potential to also become useful novel antibiotic peptides.

In conclusion, we identified single- and double-substituted CRAMP20-33 variants that display potent broad-spectrum antimicrobial activity against several pathogens and are non-toxic against human HepG2 cells. In view of the increasing interest for AMP-based drugs as new antimicrobials, these findings support the clinical potential of the described LL-37/CRAMP20-33-derived peptides as novel antimicrobial drugs.

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