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Selective cytotoxic effect against the MDA-MB-468 breast cancer cell line of the antibacterial palindromic peptide derived from bovine lactoferricin†

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The cytotoxic effect against the breast cancer cell line MDA-MB-468 of the palindromic peptide LfcinB $(21–25)_{Pal}$: ^{1}RWQWRWQWR^{9} and its analogous peptides, obtained *via* alanine scanning, was evaluated. The results indicate that the palindromic peptide exhibited a concentration-dependent cytotoxic effect against this cell line. The cytotoxic effect of the palindromic peptide was fast and selective and was sustained for up to 48 h of treatment. MDA-MB-468 cells treated with the palindromic peptide exhibited severe cellular damage, acquiring rounded forms and shrinkage, a behavior typical of apoptotic events. The analogous peptides exhibited fewer cytotoxic effects than the original palindromic peptide, suggesting that the substitution of any amino acid with alanine diminishes the cytotoxic effect. The Arg and Trp residues proved to be the most relevant for the cytotoxic effect; the analogous peptides with substitutions of Trp with Ala did not induce a change in cellular morphology, while analogous peptides with substitutions of Arg or Gln with Ala induced cellular damage. Also, neither the palindromic peptide nor its analogues exerted a significant cytotoxic effect on normal fibroblasts, indicating that the peptides had a selective cytotoxic effect on cancerous cells. The peptide LfcinB $(21–25)_{Pal}$, and its analogues exhibited antibacterial activity against *E. coli* and *S. aureus* strains and a selective cytotoxic effect against the breast cancer cell line MDA-MB-468.

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Introduction

Breast cancer is one of the leading types of cancer worldwide in terms of incidence and cause of death, with approximately 2.1 million new diagnoses and about 627 000 deaths in 2018.[1] Various local and systemic treatments have been developed to treat this disease, which include chemotherapy, surgery, hormone therapy, and radiotherapy, among others. These treatments have increased the survival rate and reduced recurrence; however, the incident rate of this cancer increases up to 5% per year in developing countries.[2–4] In order to improve treatment, new therapeutic approaches based on antimicrobial peptides (AMPs) have been studied. Bovine lactoferricin (LfcinB): ^{17}FKCRRWQWRMKKLGAPSITCVRRAF^{41} is an AMP generated from bovine lactoferrin (BLF) hydrolysis caused by the gastric pepsin. LfcinB has exhibited antibacterial, antifungal, and antiviral activity, as well anticancer activity against several types of cancer.[5–8] LfcinB exhibited higher antibacterial activity than the native protein, suggesting that LfcinB is the bactericidal domain of BLF.[9] The mechanism involved in the anticancer activity of LfcinB is not entirely clear. It is believed that the selectivity for cancer cells over normal cells is in part due to electrostatic interactions between the positively-charged peptide and the negatively-charged membrane components[10,11] such as phosphatidylserine, glycosylated mucins, sialylated gangliosides, sialic acid, and heparan sulphate in cancer cells.[12]

The minimal motif with antibacterial and anticancer activity has been identified as LfcinB (20–25): RRWQWR. This sequence is amphipathic and positively charged.[13] In previous studies, it has been reported that short synthetic peptides derived from the minimal motif exhibited higher antibacterial and anticancer activity than LfcinB and BLF.[14] Furthermore, it has been observed that palindromic peptides derived from LfcinB have exhibited significant antibacterial activity against Gram-positive

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and Gram-negative bacteria.[15] The peptide LfcinB $(21–25)_{Pal}$ (^{1}RWQWRWQWR^{9}) is derived from the minimal motif RRWQWR,[16] in which the WQW sequence is flanked by Arg residues in a palindromic arrangement. This peptide has exhibited higher antibacterial activity than LfcinB and its minimal motif on *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *S. enteritidis* ATCC 13076 and *P. aeruginosa* ATCC 27853.[17,18] Furthermore, this palindromic peptide exhibited a synergistic antibacterial effect with vancomycin[19] and a cytotoxic effect against oral squamous cell carcinoma (OSCC) CAL-27 and SCC-17.[20]

In the present paper, the palindromic peptide LfcinB $(21–25)_{Pal}$ and its analogues (alanine scan) were synthesized *via* solid-phase peptide synthesis (SPPS) and purified and characterized using RP-HPLC and MALDI-TOF MS. The peptide's antibacterial activity against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 and cytotoxic effect against MDA-MB-468 breast cancer cell line and young fibroblasts primary cell culture were assessed. Our results allowed us to establish that the peptide LfcinB $(21–25)_{Pal}$ exhibits a significant and selective cytotoxic effect against MDA-MB-468 cells. It was possible to identify the fundamental amino acids responsible for its antibacterial activity against Gram-positive and Gram-negative bacteria. Also, for cytotoxic activity against breast cancer cell line MDA-MB-468 it was established that there is not critical amino acid, instead is the alternance of charged and hydrophobic residues which gives the palindromic peptide an effect. These results allow us to demonstrate the effect of the peptide sequence on the biological activity presented and therefore serve as a starting point for the design of new and improved peptides that present enhance activity.

Results and discussion

Peptide synthesis

Peptide LfcinB $(21–25)_{Pal}$ (^{1}RWQWRWQWR^{9}) and its analogues were synthesized through the SPPS-Fmoc/*t*Bu strategy, purified by means of RP-SPE, and characterized using RP-HPLC and MALDI-TOF MS. The peptide synthesis was fast and efficient, and neither amino acid coupling nor Fmoc group removal reactions proved to be difficult. All purified peptides showed one main peak, with chromatographic purity between 85% and 95%. MALDI-TOF MS analysis showed that in all cases the purified peptide corresponded to the ratio m/z of the expected $[M + H]^{+}$ (Table 1 and ESI, S1†).

In the chromatographic profile of peptide LfcinB $(21–25)_{Pal}$ there is a principal signal at retention time (t_R) of 6.4 min with a purity of 91%; when Arg (side chain charged positively) was replaced by Ala (side chain aliphatic), the t_R of analogous peptides A1, A5, and A9 was higher than the t_R of LfcinB $(21–25)_{Pal}$, indicating that these peptides are more hydrophobic than the original peptide (Table 1). The analogous peptides A2, A4, A6, and A8 (substitution of Trp with Ala) exhibited a t_R lower than the original peptide, suggesting that the Trp side chain is more hydrophobic than the Ala side chain. When Gln was replaced with Ala in peptides A3 and A7, the t_R was similar to the t_R of the original peptide, indicating that the change does not affect the polarity of the peptide (Table 1).

The differences observed in the t_R of analogous peptides reflex differences in their physicochemical properties (net charge, polarity, hydrophobicity, and amphipacity) associated with the substitution of Arg, Trp, or Gln with Ala in the LfcinB $(21–25)_{Pal}$ sequence. In addition, the functional groups of the N-terminal (amine group) and C-terminal (amide group) ends could be responsible for the differences between analogues with the same substitutions at a different position of the sequence.

Peptide antibacterial activity

The antibacterial activity of the palindromic peptide LfcinB $(21–25)_{Pal}$ and its analogues against a Gram-negative strain of *E. coli* ATCC 25922 and a Gram-positive strain of *S. aureus* ATCC 25923 was evaluated (Table 1). It was found that the palindromic peptide LfcinB $(21–25)_{Pal}$ exhibited significant antibacterial activity against *E. coli* strain (MIC = 34 μM); this result is similar to previous studies, which reported similar MIC values for this peptide in the same strain.[18,23,30] The palindromic peptide exhibited similar antibacterial activity to those exhibited by the peptide containing the minimal motif against *E. coli* (JM-109) strain.[31]

Peptides A1, A5, and A9 exhibited lower antibacterial activity against *E. coli* ATCC 25922 than the palindromic peptide, showing that the positive charge of Arg residue is critical for its antibacterial activity. The net charge of the palindromic peptide is +4, while when Arg was replaced by Ala, the net charge of these analogous peptides A1, A5, and A9 was +3, and their antibacterial activity decreased to the MIC values 71, 143, and 143 μM, respectively.

This behaviour suggests that the decrease in antibacterial activity could be related to the loss of one positive charge, which mainly affects the net charge, polarity, and amphipathicity of the sequence. Furthermore, the substitution of any amino acid of the ^{2}WQW^{4} motif with Ala (A2, A3, and A4 peptides) causes complete loss of antibacterial activity against the *E. coli* strain, indicating that these amino acids are fundamental for the antibacterial activity against this Gram-negative strain. On the other hand, it was found that the substitution of any amino acid of the ^{6}WQW^{8} motif with Ala in the C-terminal region (A6, A7, and A8 peptides) did not significantly affect the antibacterial activity. Moreover, the replacement of the Gln^{8} by Ala (peptide A8) located in the C-terminal region led to increased activity, with a MIC of 17.5 μM. This shows that changes in the two WQW motifs of the sequence affect the antibacterial activity against the *E. coli* strain in different ways, any change in the motif located near the N-terminal region being critical.

Otherwise, the analogous peptides (except A1) and the original peptide exhibited similar antibacterial activity against *S. aureus* ATCC 25923, suggesting that there were no critical residues in the antibacterial activity against this Gram-positive strain. Interestingly, the A1 peptide exhibited higher antibacterial activity against *S. aureus* ATCC 25923 than the original peptide, suggesting that the replacement of the Arg^{1} with Ala (A1: ARWQWRWQWR) increased the antibacterial activity

Table 1 Analytical characterization and antibacterial activity of synthesized LfcinB (21–25)$_{Pal}$ and its derived peptides

		RP-HPLC		MALDI-TOF MS m/z $[M + H]^+$		*E. coli* ATCC 25922	*S. aureus* ATCC 25923
Peptide code	Sequence	t_R (min)	Purity (%)	Theor.	Exp.	MIC/MBC (μM)	
LfcinB (21-25)$_{Pal}$	**^{1}R W Q W R W Q W R^{9}**	**6.4**	**91**	**1485.8**	**1486.8**	**34/67**	**135/135**
A1	**A** W Q W R W Q W R	6.6	85	1400.7	1400.5	71/143	71/71
A2	R **A** Q W R W Q W R	5.8	93	1370.7	1371.2	146/>146	146/>146
A3	R W **A** W R W Q W R	6.4	88	1428.7	1428.8	140/140	140/ND
A4	R W Q **A** R W Q W R	5.6	95	1370.7	1369.6	146/>146	146/>146
A5	R W Q W **A** W Q W R	6.7	92	1400.7	1401.9	71/143	143/>143
A6	R W Q W R **A** Q W R	5.6	85	1370.7	1371.7	37/73	146/<146
A7	R W Q W R W **A** W R	6.5	89	1428.7	1429.5	18/35	140/ND
A8	R W Q W R W Q **A** R	5.7	90	1370.7	1371.3	37/73	146/>146
A9	R W Q W R W Q W **A**	6.7	90	1400.7	1401.5	143/>143	143/143

against this Gram-positive strain. These results suggest that the action mechanism of the palindromic peptide in Gram-positive strains is different from that for the Gram-negative strain.

Our results are in agreement with previous reports, which proposed that the antibacterial activity of the peptide LfcinB (21–25)$_{Pal}$ is due to the amino acid sequence,[17] it has a net charge of +4, and the alternation of Arg and Trp residues confers amphipathic properties.[16,24] Furthermore, it has been shown that AMPs with symmetric sequences exhibit higher antibacterial activity than similar AMPs with asymmetric sequences.[25]

Additionally, a severe loss of antibacterial activity against *E. coli* 25 922 also was observed when Trp^{22} or Trp^{24} residues were changed to Ala in the peptide LfcinB (18–32): ^{18}KCRRWQWRMKKLGAP^{32}.[24] In a way similar to our results, all alanine-modified analogues of peptide LfcinB (18–32) exhibited reduced antibacterial activity against *S. aureus* 25 923.[24] Our results and those of other authors suggest that residues Trp^{22} and Trp^{24} are essential for the antibacterial activity against certain strains.[12,26] It has been suggested that the Trp side chain is involved in the interaction of the peptide with the lipid membrane, causing membrane disruption that leads to cellular lysis. Also, it has been suggested that Trp is involved in the internalization of the peptide for reaching intracellular targets.[31,32] As well it is interesting that amphiphilic peptides containing charged and hydrophobic residues like our peptides not only can be used as a potent antimicrobial, but they are also great molecules which can be used in other delivery materials such as hydrogels[46] which maintain its polycationicity, generate membrane disruption and makes it difficult to gain resistance.[47]

In similar way to previous results the palindromic peptide and their analogues exhibited higher antibacterial activity against *E. coli* strain compared with those observed in *S. aureus* strain.[19] In this work was possible to identify the critical amino acids in the antibacterial activity against *E. coli* and *S. aureus* strains, interestingly as we study the primary structure of LfcinB (21–25)$_{Pal}$ we were able to find peptides with higher antibacterial activity as A1 against the Gram-positive strain and A7 against the Gram negative strain.

Cytotoxic assays

The cytotoxic effect of peptide LfcinB (21–25)$_{Pal}$ and its analogues against the breast cancer cell line MDA-MB-468 and young human fibroblast primary cell culture was evaluated. The cytotoxic effect of the palindromic peptide LfcinB (21–25)$_{Pal}$ against MDA-MB-468 cells was significant (cell viability of 23%) for 2, 24, and 48 h of treatment. The cytotoxic effect of the palindromic peptide was fast and concentration dependent and was sustained for up to 48 h of treatment, $IC_{50} = 75$ μg mL^{-1} (49 μM). When young human fibroblast primary cells were treated with the palindromic peptide LfcinB (21–25)$_{Pal}$, there was no cytotoxic effect at the peptide concentrations evaluated for the three treatment times, $IC_{50} > 75$ μg mL^{-1} (135 μM). This suggests that the cytotoxic effect of the palindromic peptide was selective for human breast cancer cells in all the evaluated concentrations for all treatment times. The selective cytotoxic effect observed indicate that palindromic peptide doesn't affect the cell viability of the fibroblasts which are a cell type found in breast and which has even been shown to have a close relationship with the cells within the tumour microenvironment of various types of cancer, including breast cancer.[48–50]

The cytotoxic effect of the palindromic peptide against breast and oral cancer cell lines is according with other reports that showed that BLF, LfcinB and other peptides containing the minimal motif exhibited cytotoxic effect *in vitro* and/or *in vivo* assays against different cancer types included breast cancer.[9,18,20,22,33–40]

In similar way to palindromic peptide, synthetic short peptides derived of hLfcin containing Trp and Arg residues reported to be active against *E. coli* and bacterial lipid model systems, also exhibited selective cytotoxic effect against primary melanoma cells (SBcl-2) and metastatic lesions (WM164), a melanoma cell line (A375), a rhabdomyosarcoma cell line (TE671), a glioblastoma cell line (U-87 mg) and did not show to be toxic for non-cancer cells as melanocytes and normal human dermal fibroblasts. The selective cytotoxic effect of these peptides was associated to PI-uptake indicating loss of cell membrane integrity and cell death.[41]

This behaviour suggests that the decrease in antibacterial activity could be related to the loss of one positive charge, which

mainly affects the net charge, polarity, and amphipathicity of the sequence.

It was found that all the analogous peptides exhibited a lower cytotoxic effect against MDA-MB-468 cells than the palindromic peptide (cellular viability 23%), the cellular viability being between 40% and 71% (Table 2). The cytotoxic effect of palindromic peptide and all the analogous peptides was similar when the cells were treated for 2 h, 24 h, or 48 h, and the cytotoxic effect was concentration-dependent in all cases (Table 2, Fig. 1, see ESI, S2†). These results allow us to infer that all the residues played an important role in the peptide's cytotoxic effect. This agrees with previous reports, which indicated that the RRWQWRMKKLG sequence is critical for the biological activity of LfcinB.[27]

The importance of Arg, Trp, and Gln for the cytotoxic effect against breast cancer cells lies in the fact that these amino acids are what give the peptide the amphipathic properties and allow it to establish the electrostatic interaction with the cell membrane. It has also been proposed that Trp residues are the key for inducing peptide internalization.[26,28,29] These results are in accordance with the fact that the LfcinB interacts with the cell surface through electrostatic attraction between the positive charges of the peptide and the negative charges on the cell surface, and the substitution of any amino acid of peptide LfcinB $(21–25)_{Pal}$ affects both the amphipaticity and the net charge of the sequence.

The morphologic changes in MDA-MB-468 and fibroblast cells induced by the peptide LfcinB $(21–25)_{Pal}$ and its analogues were evaluated. The MDA-MB-468 cells treated with the peptide LfcinB $(21–25)_{Pal}$ exhibited dramatic morphological changes, rounded forms and shrinkage (Fig. 2), which has been related to apoptotic events. While untreated cells showed the morphological characteristics of typical MDA-MB-468 cells in a stellate shape, the morphological changes in breast cancer cells induced by the palindromic peptide could be associated with the cytotoxic effect observed in MTT assays. In a similar way,

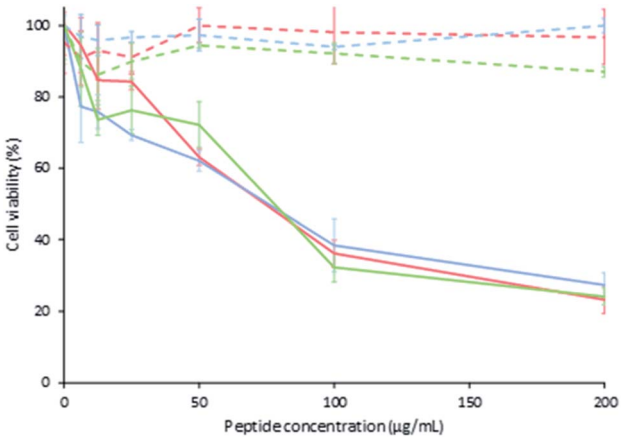

Fig. 1 Cytotoxic effect by MTT of LfcinB (21–25)Pal peptide on MDA-MB-468 in continuous line and young fibroblast cells in discontinuous line incubated at 2 h (red), 24 h (blue) and 48 h (green) ± S.D. ($n = 3$).

when the MDA-MB-468 cells were treated with the peptides A1, A3, A5, and A7 (substitution of Arg or Gln with Ala), morphological changes were observed, acquiring rounded forms. In a previous report, we showed that the tetrameric peptide LfcinB $(20–25)_4$, which contains the minimal motif RRWQWR, exhibited a selective and fast cytotoxic effect against MDA-MB-468 cells, which is associated with the apoptotic process.[22]

It has been suggested that BLF, cyclic LfcinB, and linear LfcinB exerted antitumor activities through activating diverse signalling pathways, including p53, apoptosis, and angiopoietin signalling, which could induce cellular morphological changes.[35] Furthermore, LfcinB causes severe damage in cytoplasm and mitochondria membranes of neuroblastoma cells.[29]

When the breast cancer cells were treated with peptides A2, A4, A6, and A8 (substitution of Trp with Ala), significant morphological changes associated with the cytotoxic effect were not observed. On the other hand, young human fibroblasts

Table 2 Cytotoxic effect of peptides against MDA-MB-468 and young fibroblast cells. The peptide concentration was 200 μg mL^{-1}, ± SD ($n = 3$) ANOVA post hoc Sidak

	Cellular Viability (%)					
	MDA-MB-468 cells			Fibroblast cells		
Peptide code	2 h	24 h	48 h	2 h	24 h	48 h
LfcinB$(21–25)_{Pal}$	**23 ± 4**	**27 ± 4**	**24 ± 2**	**97 ± 5**[a]	**100 ± 2**[a]	**92 ± 2**[a]
A1	53 ± 4	48 ± 5	51 ± 2	99 ± 2[a]	100 ± 1[a]	92 ± 2[a]
A2	62 ± 4	63 ± 4	58 ± 6	100 ± 2[a]	94 ± 2[a]	87 ± 2[a]
A3	45 ± 7	40 ± 6	43 ± 6	100 ± 3[a]	91 ± 5[a]	86 ± 4[a]
A4	59 ± 9	52 ± 1	51 ± 9	100 ± 4[a]	79 ± 4[a]	66 ± 2[c]
A5	71 ± 8	62 ± 7	64 ± 4	99 ± 1[a]	81 ± 1[b]	82 ± 1[a]
A6	55 ± 6	49 ± 5	52 ± 5	100 ± 2[a]	88 ± 2[a]	79 ± 4[a]
A7	59 ± 6	57 ± 3	56 ± 1	96 ± 5[a]	100 ± 1[a]	79 ± 5[a]
A8	69 ± 5	62 ± 5	61 ± 6	100 ± 1[a]	94 ± 1[a]	85 ± 4[a]
A9	64 ± 7	55 ± 6	65 ± 7	100 ± 4[a]	98 ± 3[a]	100 ± 3[a]

[a] Statistic significant differences with regard to the effect on MDA-MB-468 cells at same incubation time $p < 0.0001$. [b] Statistic significant differences with regard to the effect on MDA-MB-468 cells at same incubation time $p < 0.001$. [c] Statistic significant differences with regard to the effect on MDA-MB-468 cells at same incubation time $p < 0.05$.

treated with the peptide LfcinB $(21–25)_{Pal}$ or its analogous peptides did not exhibit morphological changes (Fig. 2). The morphology of treated and untreated young human fibroblasts was similar, which is in accord with the results of the MTT assays, confirming that the cytotoxic effect of the evaluated peptides is selective for breast cancer cells. These results indicate that the cytotoxic effect of the peptides A2, A4, A6, and A8 is lesser than original peptide and is not associated with significantly morphological changes suggesting that the change of one Trp residue affects the activity of the peptide. These results are agreed with reports that showed that Trp residues in LfcinB has been associated with membrane disruption and/or internalization which could induces cellular severe damage.[31,32]

The selective cytotoxic effect of palindromic peptide and their analogous peptides is related with the cellular damage observed in breast cancer cells MDA-MB-468. Fundamental differences exist between the cell membranes of malignant cells and normal cells that likely account for the ability of certain AMPs to kill cancer cells. The electrostatic interactions between cationic peptides and anionic cell membrane components as phospholipids and *O*-glycosylated mucin can be the major factor in the selective killing of cancer cells. The selectivity of palindromic peptide by cancer cells has been attributed to the membrane fluidity of cancer cells because is greater than that of untransformed cells, being their amphipathic nature the main responsible of the enhanced lytic activity through membrane destabilization.[42]

Interesting, that the all peptides exhibited significantly selective cytotoxic effect against breast cell cancer the MDA-MB-468, suggesting that punctual changes in the sequence diminished the cytotoxic effect but it not was suppressed completely. By other hand, it can be observed that the results obtained by analogous peptides in antibacterial assays was unlike of those registered for cytotoxic assays. This behaviour is according with the fact that cytotoxic effect of palindromic peptide could be related with an independent mechanism to the one established in bacterial strains. In order to determine if the antibacterial and anticancerogenic activities are related with changes in secondary elements structural, CD Spectra were recorded for each peptide.

The CD spectrum of peptide LfcinB $(21–25)_{Pal}$ exhibits a pattern with a positive band at 220–230 nm and a negative band at 195–210 nm, which suggests a random coil structure. The same pattern is observed for all analogous peptides, suggesting that the antibacterial activity and the selective cytotoxic effect against breast cancer cell line MDA-MB-468 isn't related to secondary structural elements (ESI, S3†).

The CD results are according with previous reports that showed that CD spectra of motif RRWQWR at 10 μM in 10 mM Tris pH 7.4 is characteristic of random coil pattern.[43] The random coil structure observed in CD spectra of palindromic peptide is coherent with the CD spectra of BLF, LfcinB and

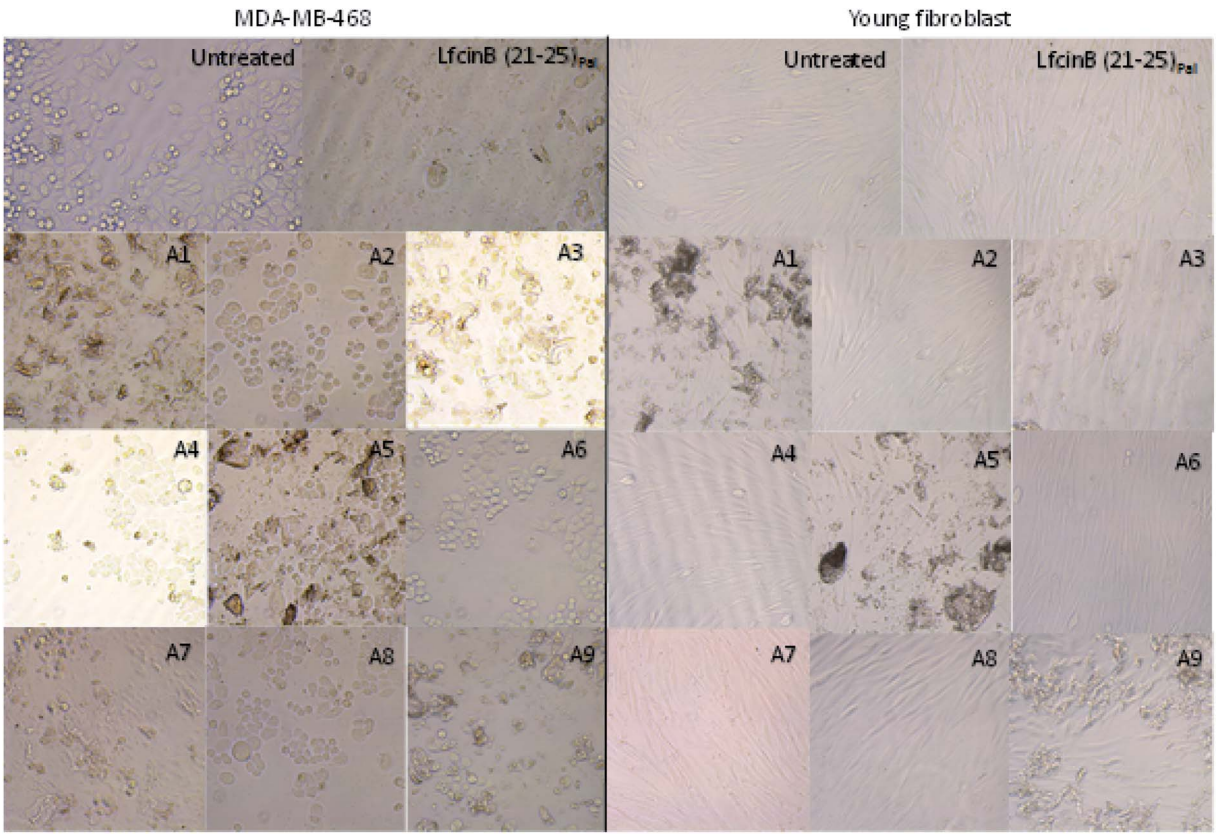

Fig. 2 Microphotographs by contrast microscopy of the MDA-MB-468 and young fibroblast cells untreated, treated with LfcinB $(21–25)_{Pal}$ and analogous peptides at 134,61 μM by 24 h.

synthetic peptides LfcinB (17–31), LfcinB $(20–25)_4$ and LfcinB $(20–25)_{Cyc}$, suggesting that native protein, the LfcinB and other short peptides containing the RRWQWR motif do not have secondary structural elements of Beta sheet and/or Helix conformation.[44] Our results agree with previous reports of CD spectra which indicate that the secondary structure of LfcinB is independent from the environment conditions and the helical population is lower than 10%, the remaining being almost equally distributed between the unstructured and B-sheet structure.[45]

The evaluated cytotoxic effect against breast cancer cell line MDA-MB-468 was selective, fast, concentration dependent, and stable up to 48 h, and could be associated with the apoptotic pathway. Our results confirm that the antibacterial and anticancer activity of a palindromic peptide mainly depends on its amino acid and symmetry sequence, net charge, and amphipaticity. The peptide LfcinB $(21–25)_{Pal}$ could be considered to be a promising candidate for developing new therapeutic agents against breast cancer.

Material and methods

Solid-phase peptide synthesis

The peptides were synthesized using manual SPPS-Fmoc/*t*Bu in accordance with Vargas *et al.*[19] Briefly, 100 mg of Rink amide resin (0.46 meq g^{-1}) was treated with DCM for 2 h at room temperature (RT). (i) Fmoc group removal was carried out through treatment with 2.5% 4-methylpiperidine in DMF for 10 min at RT with constant stirring (2×). (ii) For the coupling reaction, Fmoc-amino acids (0.21 mmol) were pre-activated with DCC/6-Cl–HOBt (0.20/0.21 mmol) in DMF for 15 min at RT with stirring. Afterward, the pre-activated amino acid was added to the resin or resin-peptide and the reaction mixture was stirred for 2 h at RT. The Fmoc group removal and the coupling reactions were monitored with the Kaiser test. (iii) Side-chain deprotection reactions and peptide separation from the resin were carried out by means of treatment of resin-peptide with a solution containing TFA/water/TIPS/EDT (93/2/2.5/2.5 v/v/v) for 8 h with stirring at RT. (iv) Crude peptides were precipitated by treatment with cool ethyl ether and were washed five times with cool ethyl ether, and then the solid was dried at RT.

Reverse-phase HPLC RP-HPLC

The peptides were characterized by means of RP-HPLC chromatography in accordance with Insuasty *et al.*[21] Briefly, the analysis was performed on a Merck Chromolith® C18 (50 mm × 4.6 mm) column using an Agilent 1200 liquid chromatograph (Omaha, NE, USA) with UV-Vis detector (210 nm). 10 μL of peptide solution (1 mg mL^{-1}) was injected, and a linear gradient was applied from 5% to 50% solvent B (0.05% TFA in ACN) in solvent A (0.05% TFA in water) for 8 min at a flow rate of 2.0 mL min^{-1} at RT.

Peptide purification

The peptides were purified using solid-phase extraction columns following the methodology reported by Insuasty *et al.*[21] Briefly, SPE columns (SUPELCO LC-18 with 2.0 g resin) were activated with 30 mL of solvent B and equilibrated with 30 mL of solvent A according to the manufacturer's recommendations. Crude peptides were passed through the column, and a gradient of solvent B (5–50%) was used for their elution. Collected fractions were analyzed using RP-HPLC (as described above), and fractions that contained the pure peptide were collected and lyophilized.

MALDI-TOF MS

The peptide (1 mg mL^{-1}) was mixed with 2,5-dihydroxybenzoic acid or sinapinic acid (1 mg mL^{-1}) at 2 : 18 (v/v), and 1 μL was seeded on the steel target. The experiment was performed on an Microflex TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in reflectron mode, using an polished steel target (Bruker Daltonics, Bremen, Germany) laser: 500 shots and 25–30% power.

Circular dichroism

The CD spectrum of the peptides was recorded following the methodology described by Huertas *et al.*[17] Briefly, the peptide (0.2 mM) was dissolved in 2,2,2-trifluoroethanol (30%) aqueous solution and then analyzed in a spectropolarimeter Jasco J-810, between 190 and 260 nm at 25 °C in a quartz cuvette with a 1 cm path length. The result is the average of three scans taken at 20 nm min^{-1} with a spectral bandwidth of 1 nm.

Antibacterial activity assays

The antibacterial activity of peptides against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 strains was evaluated in accordance with Vargas *et al.*[18] Briefly, the minimum inhibitory concentration (MIC) was determined by a microdilution assay (CLSI) in a 96-well microplate. 90 μL of peptide (200, 100, 50, 25, 12.5 and 6.2 μg mL^{-1}) and 10 μL of inoculum (5×10^6 CFU mL^{-1}) were added and incubated for 24 h at 37 °C, and afterward the absorbance was measured at 620 nm. The minimum bactericidal concentration (MBC) was determined by taking samples from each well where there was no visible bacterial growth, which were spread on Mueller Hinton agar boxes (AMH) and incubated for 24 h. The MBC was chosen as the lowest concentration where no CFU was evidenced. Each analysis was performed in duplicate ($n = 2$).

MTT assays

Cytotoxicity assays were performed as previously described in Rodriguez *et al.*[22] Briefly, breast cancer cell line MDA-MB-468 (ATCC® HTB-132™) cells or young fibroblast primary cells (100 μL; 1×10^4 cells per well) were seeded in 96-well flat-bottom tissue culture treated plates and were incubated at 37 °C in a 5% CO_2 humidified atmosphere for 14 h, allowing for cell adhesion. Then the media was removed and 50 μL of supplemented RPMI medium (10%) and 50 μL of peptide (200, 100, 50, 25, 12.5 and 6.25 μg mL^{-1}) were added, and the final FBS concentration was 5%. The plates were incubated for 2, 24, and 48 h at 37 °C in a 5% CO_2 humidified atmosphere (physiologic

pH), and cell viability was determined using the MTT assay. For this, 10 μL of MTT solution (5 mg mL^{-1}) was added to each well, and the plates were incubated for 4 h at 37 °C. Formazan crystals were dissolved in DMSO (100 μL), and the absorbance (570 nm) was measured using a Bio-Rad 680 microplate reader ($n = 3$).

Microscopy assays

Prior to the addition of MTT to the cytotoxicity assays, photographic recording was performed *via* phase-contrast microscopy of the cells treated with the peptides and the untreated ones using a Leica DMi microscope at 20×.

Conclusions

In the present work, 9 peptides derived from an Alanine scan of the LfcinB $(21–25)_{Pal}$ peptide were designed and synthesized in order to determine the critical amino acids related to their secondary structure and biological activity against both bacteria and cancer cells. It was evidenced that all the peptides presented a random coil type secondary structure so that the changes in their primary structure do not affect the secondary structure elements. Regarding antibacterial activity, for *S. aureus* 25 923 it was shown that changes in the sequence generated total loss of activity, determining that all amino acids are relevant to exert their bacteriostatic and bactericidal effect. The data obtained for *E. coli* ATCC 25922 showed that the change in the amino acids relating to the $^{2}WQW^{4}$ motif generate total loss of their antibacterial activity, being these so-called critics. These results allow us to suggest that the mechanism of action exerted may differ on Gram-positive and Gram-negative bacteria.

About the cytotoxicity exerted against the cell line derived from breast cancer MDA-MB-468, it was shown that the palindromic peptide has a concentration-dependent effect, fast and maintained for up to 48 h after a single administered dose. In addition, it was observed that under none of the concentrations and times evaluated was the cell viability of the primary culture of fibroblasts affected, which could indicate that the effect of the palindrome is selective for cancer cells. When evaluating the effect of the analogues, it could be established that the change in any of the amino acids generated a decrease in the cytotoxic effect compared to that exerted by the palindrome, which indicates that this cytotoxicity is not given by critical amino acids but is alternation of charged and hydrophobic residues given by the palindromic arrangement which generates this effect.

These results allow us to demonstrate that the antibacterial and cytotoxic effect is influenced to a greater extent by changes in the primary structure than in the secondary structure of the LfcinB $(21–25)_{Pal}$ peptide, in addition to evidencing that depending on the biological model the effect of the sequence is differential. Additionally, in an interesting way peptides A7 (change in Gln residue at the C-terminal) and A1 (change in Arg residue at the N-terminal) derived from the palindrome shown an improved activity for *E. coli* and *S. aureus*, respectively. In this way, our results not only generate a greater understanding of the LfcinB $(21–25)_{Pal}$ peptide, but also represent a starting point for the design of novel and improve peptides that can enhance its activity. The palindromic peptide could be considered as promissory candidate to develop new therapeutically options based in synthetic peptides.

Conflicts of interest

There are no conflicts to declare.

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