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Changes in Length and Positive Charge of Palindromic Sequence RWQWRWQWR Enhance Cytotoxic Activity against Breast Cancer Cell Lines

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Cite This: *ACS Omega* 2023, 8, 2712−2722

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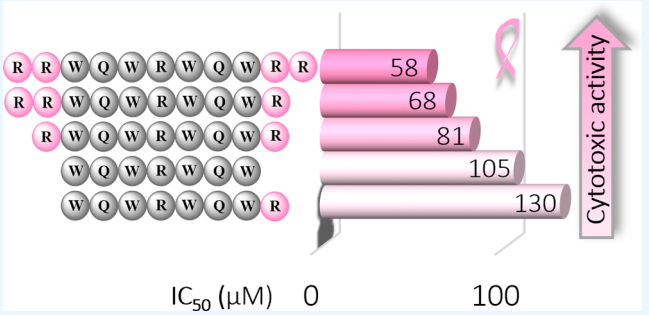
ABSTRACT: Breast cancer is one of the main causes of premature death in women; current treatments have low selectivity, generating strong physical and psychological sequelae. The palindromic peptide R-1-R (RWQWRWQWR) has cytotoxic activity against different cell lines derived from cancer and selectivity against noncancerous cells. To determine if changes in the charge/length of this peptide increase its activity, six peptides were obtained by SPPS, three of them with addition of Arg at the N, C-terminal or both and three with deletion of Arg at the N, C-terminal or both. The cytotoxic and selective activities were evaluated against MCF-7, MDA-MB-231, and MCF-12 cell lines and fibroblast primary cell culture, evidencing that the RR-1-R peptide with the inclusion of Arg in the N-terminal end maintained selectivity and increased cytotoxicity against lines derived from breast cancer. The effect of this addition regarding the type of induced cell death was evaluated by flow cytometry, showing very low rates of necrosis and a significant majority of apoptotic events with activation of both Caspase 8 and Caspase 9. This work allowed us to find a modification that generates a peptide with greater cytotoxic effects and can be considered a promising molecule for other approaches to improve anticancer peptides.



INTRODUCTION

Cancer is an escalating health issue worldwide as the second cause of premature death; according to the World Health Organization (WHO), it may surpass cardiovascular diseases as the leading cause in the next few years.[1] Since 2020, breast cancer is the most common type of cancer; more than 2 million people are diagnosed every year with this disease and nearly 700.000 people die from it.[2] In women, this type of cancer accounts for 50% of all new cases, having the greatest incidence, mortality, and prevalence and representing a major health challenge.[3]

Even though in the past 30 years the death rate has declined in 42%, the efforts made to strengthen the detection and treatment system have not been sufficient.[4] Current treatment for nonmetastatic cancer is surgical resection along with postoperative radiation or systemic treatment; depending on the subtype, endocrine therapy, immunotherapy, or chemotherapy is chosen; for metastatic breast cancer, a systemic treatment is employed. Although several advances in improving these treatments have been made, there are still long-term cosmetic, functional, and psychological sequels such as hot flashes, arthralgias, myalgias, nausea, myelosuppression, and uterine cancer.[5]

New alternatives such as antimicrobial peptides (AMPs) are being evaluated due to their fast interaction, reduced toxicity, good solubility, and ease of synthetic modification.[6] Some of these AMPs are cationic, which interact electrostatically with anionic components of the bacterial cell membrane, and since cancerous cells also have a negative cell membrane due to the impaired phospholipid in the external cell membrane and a higher expression of O-glycosylated mucins, a cytotoxic effect against cancerous cells is also evaluated.[7] Bovine lactoferricin (LfcinB) is a 25 amino acid AMP derived from bovine lactoferrin protein that has exhibited cytotoxic effects against different microorganisms like Gram-positive and Gram-negative bacteria and *Candida* spp.[8] and also against liver,

Received: November 15, 2022
Accepted: December 21, 2022
Published: January 5, 2023

https://doi.org/10.1021/acsomega.2c07336
ACS Omega 2023, 8, 2712−2722

Table 1. Characterization of Modified Peptides Derived from Palindromic Sequence RWQWRWQWR

code	sequence	net charge	RP-HPLC		ESI-MS m/z $[M + H]^+$	
			t_R (min)	purity (%)	theoretical	exp.
R-1-R	RWQWRWQWR-NH_2	+4	6.3	92	1485.75	1485.83
RR-1-RR	RRWQWRWQWRR-NH_2	+6	5.9	97	1799.09	1799.02
R-1-RR	RWQWRWQWRR-NH_2	+5	61	93	1642.91	1642.90
RR-1-R	RRWQWRWQWR-NH_2	+5	6.2	93	1640.93	1642.89
1	_WQWRWQW_-NH_2	+2	7.1	96	1173.56	1172.54[a]
R-1	RWQWRWQW_-NH_2	+3	7	92	1330.53	1330.67
1-R	_WQWRWQWR-NH_2	+3	6.6	88	1330.53	1330.67

[a] m/z obtained by MALDI-TOF.

Table 2. Cytotoxic Effect of Modified Peptides Derived from Palindromic Sequence RWQWRWQWR

code	peptide sequence	IC_{50} $\mu g\ mL^{-1}$ (μM)				hemolysis[b]
		MCF-7	MDA-MB-231	MCF-12	fibroblasts	
R-1-R	RWQWRWQWR-NH_2	120(81)	154(103)	>200(>134[a])	>200(>134[a])	6.6%
RR-1-RR	RRWQWRWQWRR-NH_2	106(58)	121(67)	199(111)	>200(>111[a])	5.9%
R-1-RR	RWQWRWQWRR-NH_2	200(122)	156(95)	124(76)	>200(>122[a])	8.1%
RR-1-R	RRWQWRWQWR-NH_2	112(68)	120(73)	>200(>121[a])	>200(>121[a])	9.1%
1	_WQWRWQW_-NH_2	124(105)	>200(>170)	200(170)	61(54)	8.3%
R-1	RWQWRWQW_-NH_2	78(58)	>200(>150[a])	>200(>150[a])	>200(>150[a])	14.2%
1-R	_WQWRWQWR-NH_2	182(130)	>200(>150[a])	>200(>150[a])	>200(>150[a])	11.3%

[a] IC_{50} value could not be calculated since at maximum concentration, the viability was over 50%. [b] Percentage of hemolysis at the IC_{50} determined in MTT assays in MCF-7 cells.

lung, colon, fibrosarcoma, leukemia, and breast cancer cell lines. It has been suggested that LfcinB binds to the cell membrane through electrostatic interactions, leading to membrane disruption and causing cellular lysis. It has also been reported that the peptide may internalize into the cell and cause apoptosis-mediated cell death. Synthetic peptides containing the RRWQWR motif have been reported to induce cell death mediated by apoptosis or necrosis, depending on the concentration of the peptide and the degree of damage to the cytoplasmic membrane.[9−12] Its activity has been related to the minimal active motif or antimicrobial core, a six-residue peptide (RRWQWR), which has displayed similar or greater antibacterial activity than LfcinB or LF.[8] This free hexapeptide did not exert a cytotoxic effect on T-leukemia or breast cancer cells, but once it was delivered into the cell and bound to the mitochondrial membrane, it killed them in a similar way the whole peptide does.[13]

From this one, multiple peptide sequences with different modifications have been designed and synthetized. The generation of palindromic peptides from active sequences has improved the antimicrobial effect against Gram-negative bacteria while maintaining its selectivity.[14] To translate this approach to anticancer activity, we designed the palindromic sequence LfcinB(21-25)$_{Pal}$ RWQWRWQWR from the minimal motif of LfcinB; in a free form, it exerts a concentration-dependent and rapid cytotoxic effect on triple negative breast cancer cell lines MDA-MB-468 and MDA-MB-231 with IC_{50} of 72 and 135 μM, respectively. The same way against luminal breast cancer cell line MCF-7, an IC_{50} of 66 μM was registered. Its selectivity has been tested in primary fibroblasts, non-cancerous cells MCF-12, and bovine mammary cells BMEC, with minimum effect on them,[6,15] showing that the palindromic array enhanced the selective anticancer activity.

Given this successful case, we intend to test other modifications that improve the antimicrobial activity of AMPs. Some authors have hypothesized that the more cationic the peptide, the greater is the intake through the membrane;[16] others have shown that the addition or substitution of arginine (Arg) is not a guarantee of an improvement in activity against Gram-positive and Gram-negative ATCC bacteria, but the position is key to obtain it;[17] some others have tried to find the right percentage of Arg residues that will reduce MIC of already active peptides, proposing that around 30% of Arg in the sequence enhances antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas Aeruginosa,* and *Candida albicans*.[18] The poly Arg peptide (R9) has been reported to be able to deform membranes, and it is suggested that negatively charged lipids facilitate membrane translocation of cationic Arg-rich peptides. Arg has a hydrocarbon side chain containing a guanidinium (polar) group at the end, and it has been suggested that negative groups on the polar heads of lipids can stabilize charged Arg residues, causing the lipid membrane to deform and dehydrate locally.[19] In this work, we synthetized six peptides derived from the palindromic sequence LfcinB(21-25)$_{Pal}$ adding and eliminating R residues from N- and C-terminals of the sequence; then the cytotoxic activity against MCF-7 and MDA-MB-231 breast cancer cell lines was evaluated, its selectivity against MCF-12 and fibroblast cells was tested, and the type of cell death of the most promising modification was compared to the one exerted by the original peptide.

RESULTS

Peptide Synthesis. To determine if changes in the positive charge of palindromic peptide LfcinB (21-25)$_{Pal}$ would increase the cytotoxic effect against breast cancer cells while maintaining its selectivity, analogous peptides were designed by adding or removing Arg residues at the N-terminal, the C-terminal, or both ends. To accomplish this, six modified peptides were designed, synthetized by SPPS-Fmoc/*t*Bu, purified by reverse-phase HPLC (RP-HPLC), and characterized by ESI-QTOF mass spectrometry. To make easier

https://doi.org/10.1021/acsomega.2c07336
ACS Omega 2023, 8, 2712−2722

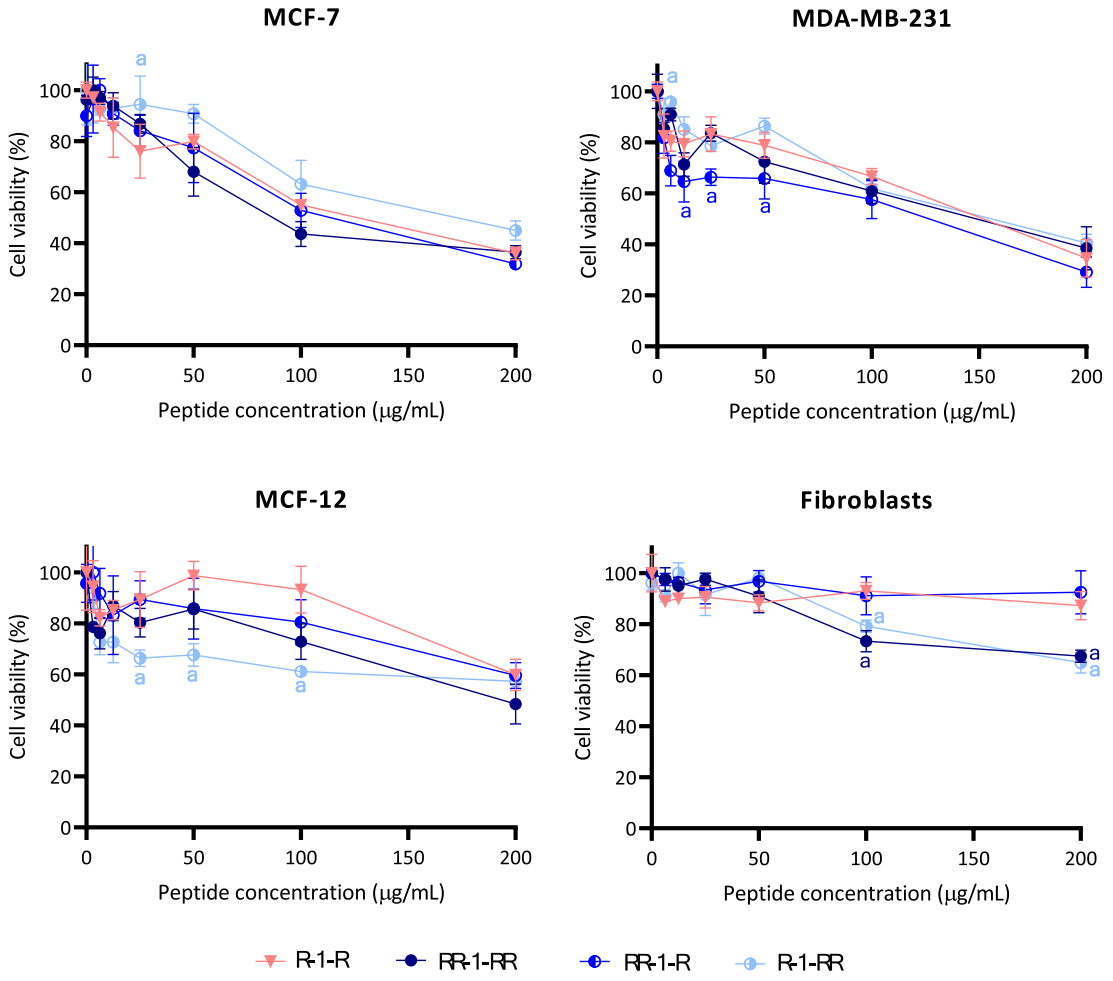


Figure 1. Cytotoxic effect of palindromic peptide R-1-R (RWQWRWQWR) and its modifications with incorporation of Arg residue at the N-terminal of RR-1-R, the C-terminal of R-1-RR, and both ends of RR-1-RR against breast cancer cells MCF-7 and MDA-MB-231 (top of the image), and normal MCF-12 and fibroblast cells (bottom of the image). The data are expressed as the mean ± SE (n = 3). [a]Statistically significant differences with palindromic peptide $p < 0.05$, ANOVA, Tuckey's multiple comparisons test.

reading, all peptides were coded having in account its central motif, WQWRWQW (coded as peptide 1), and the Arg was added around this sequence; for example, the palindromic peptide LfcinB $(21\text{-}26)_{Pal}$: RWQWRWQWR corresponds to R-1-R code and so on, Table 1. All chromatographic profiles showed a majoritarian species, indicating that the purified fraction does not have unwanted species that could affect the biological assays (Figure S1) accounting for chromatographic purities over 88% in all cases (Table 1). Alongside that, mass spectrometry spectra showed that the isotopic distribution of its masses confirmed the peptide sequences since it correlates with the expected mass of each one of them (Figure S2 and Table 1).

Cytotoxic Effect against Breast Cancer Cell Lines. Even though the effect of palindromic peptide has already been set on MCF-7 and MDA-MB-231, this new batch of peptide R-1-R was used as a control in all experiments to assess the gain or loss of anticancer activity. According to the previous reports, this new batch of palindromic peptides exhibits similar activity (Table 2) with IC_{50} values for MCF-7 (81 μM), MDA-MB-231 (103 μM), MCF-12 (>134 μM), and fibroblast (>134 μM) cells.

The addition of Arg in the N-terminal, the C-terminal, or both ends increases +1 or +2 the net charge of the peptide showing cytotoxic activity patterns similar to those of the palindromic peptide in both breast cancer cell lines, where the effect was concentration-dependent and fast, showing a decrease in cell viability near 40% at the highest concentration (Table 2 and Figure 1). Nevertheless, the IC_{50} calculated a decrease in the peptides with the addition of Arg at the N-terminal; R-1-R exhibited a toxicity of 50% of MCF-7 and MDA-MB-231 cells at concentrations of 71 and 81 μM, while RR-1-R was at 75 and 61 μM. The inclusion of Arg at only the C-terminal (R-1-RR) induced less cytotoxic activity at concentrations below 100 $\mu g\ mL^{-1}$, with an IC_{50} of 134 μM for MCF-7 and 105 μM for MDA-MB-231.

Regarding the selectivity, the incorporation of Arg at the C-terminal in RR-1-RR and in R-1-RR induced a statistically significant cytotoxic effect against MCF-12 and fibroblast cells when compared to the palindromic peptide, while the effect of the incorporation of Arg at the N-terminal in RR-1-R maintained the selectivity unchanged. These results suggest that the introduction of Arg to a sequence (net charge +5) at the N-terminal did not affect the selectivity but increased the cytotoxic effect at a lesser concentration, while with the incorporation of Arg at the C-terminal, the cytotoxicity was not affected, but selectivity was reduced. None of the four peptides

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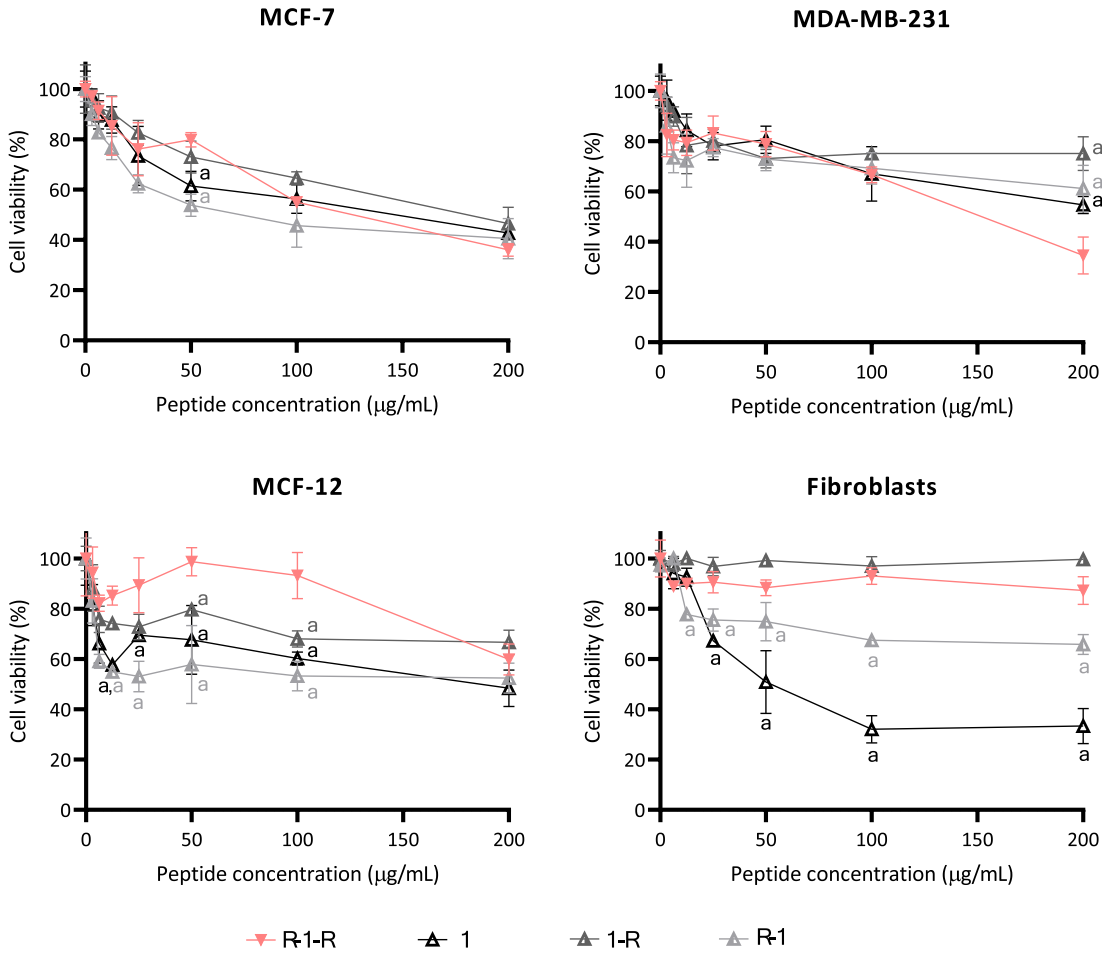

Figure 2. Cytotoxic effect of palindromic peptide R-1-R (RWQWRWQWR) and its modifications with removal of Arg residue at the N-terminal 1-R, the C-terminal R-1, and both ends 1 against breast cancer cells MCF-7 and MDA-MB-231 (top of the image), and normal MCF-12 and fibroblast cells (bottom of the image). The data are expressed as the mean ± SE (n = 3). [a]Statistically significant differences with palindromic peptide $p < 0.05$, ANOVA, Tuckey's multiple comparisons test.

Table 3. Selectivity of Modified Peptides Derived From Palindromic Sequence RWQWRWQWR[a]

	selectivity (IC_{50} noncancer cells/IC_{50} cancer cells)					
code	MCF-12/MCF-7	MCF-12/MDA-MB-231	fibroblasts/MCF-7	fibroblasts/MDA-MB-231	erythrocytes/MCF-7	erythrocytes/MDA-MB-231
R-1-R	>1.7	>1.3	>1.7	>1.3	>1.7	>1.3
RR-1-RR	**1.9**	**1.7**	>1.9	>1.7	>1.9	>1.7
R-1-RR	0.6	0.8	>1.0	>1.3	>1.0	>1.3
RR-1-R	>1.8	>1.7	>1.8	>1.7	>1.8	>1.7
1	>1.6	1.0	0.5	0.3	>1.6	>1.0
R-1	**2.6**	>1	**2.6**	**1.0**	>2.6	>1.0
1-R	>1.1	>1.0	>1.1	~1.0	>1.1	>1.0

[a]Peptides in bold are those that have a greater selectivity than the palindromic peptide.

exerted significant hemolysis at any of the evaluated concentrations (Figure S3).

The removal of Arg at both ends diminished the net charge to +2, and the 1 peptide slightly reduced the cytotoxic activity against MCF-7 and MDA-MB-231 breast cancer cell lines when compared to the palindromic. When normal fibroblasts and MCF-12 cells were treated with this modified peptide, the cytotoxic effect increased, leading to IC_{50} of 40 and 134 μM (Figure 2). A similar effect was observed in a +3 net charge peptide (R-1) when Arg was eliminated from the C-terminal only, where an increased toxicity against fibroblast and MCF-12 cells was noted, diminishing the selectivity. Also, against breast cancer cell line MDA-MB-231, the cytotoxic effect was reduced while maintaining the effect on MCF-7 cells. When the cells were treated with the 1-R peptide, the cell viability of MDA-MB-231 cells did not reach 50% at any of the concentrations used, and in MCF-7 IC_{50}, it went from 81 to 134 μM. Regarding its selectivity, in the fibroblast, cell viability was maintained at around 100%, while in MCF-12, it decreased to nearly 70% when treated with peptide concentrations from 6.25 to 200 μg mL^{-1}. With regard to the effect of these peptides on red blood cells (RBCs), the 1 peptide induced a significant hemolysis of 16% at maximum concentration, 1-R did not cause any significant damage to

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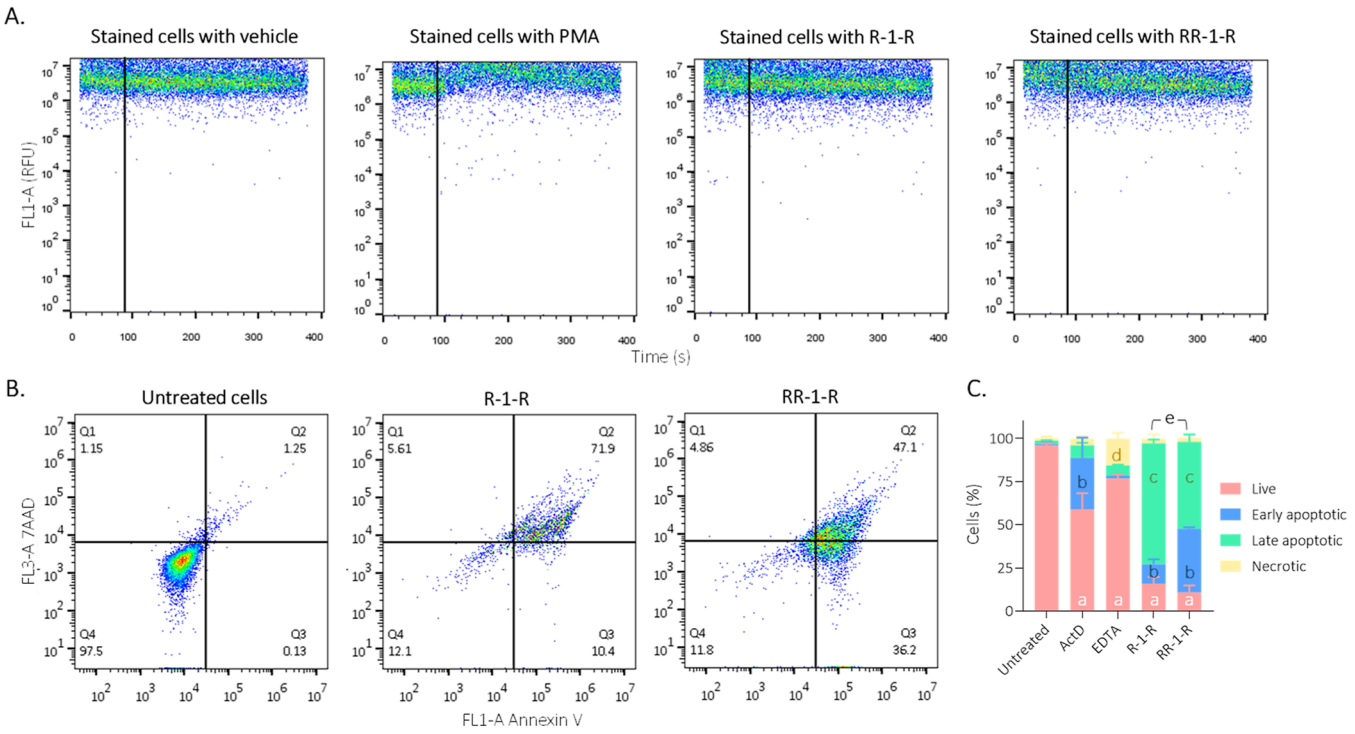

Figure 3. Representative dot plots of the cytometry assay to determine the type of cell death induced in MCF-7 cells A. Cytosolic calcium efflux measurement (n = 3). Negative control vehicle (RPMI 1640 medium), positive control PMA, R-1-R, and RR-1-R at IC_{50}. The black line indicates the time at which the treatments were administrated. B. Determination of apoptosis/necrosis in cells treated for 2 h with the IC_{50} of the peptides R-1-R and RR-1-R. Q1: Events in early apoptosis, Q2: late apoptosis, Q3: necrosis, and Q4: live events. Apoptosis control actinomycin D, necrosis control EDTA, and live control RPMI 1640 medium. C. Bar graph of the percentage of events related to live cells in early or late apoptosis or necrosis in each treatment (n = 3), ANOVA, Tukey's multiple comparisons test. [a]Statistically significant differences with live percentage of untreated cells, $p < 0.0001$. [b]Statistically significant differences with early apoptotic percentage of untreated cells, $p < 0.0005$. [c]Statistically significant differences with late apoptotic percentage of untreated cells, $p < 0.0001$. [d]Statistically significant differences with necrosis percentage of untreated cells, $p < 0.0001$. [e]Statistically significant differences with early/late apoptotic percentage of cells treated with palindromic peptide R-1-R, $p < 0.0001$.

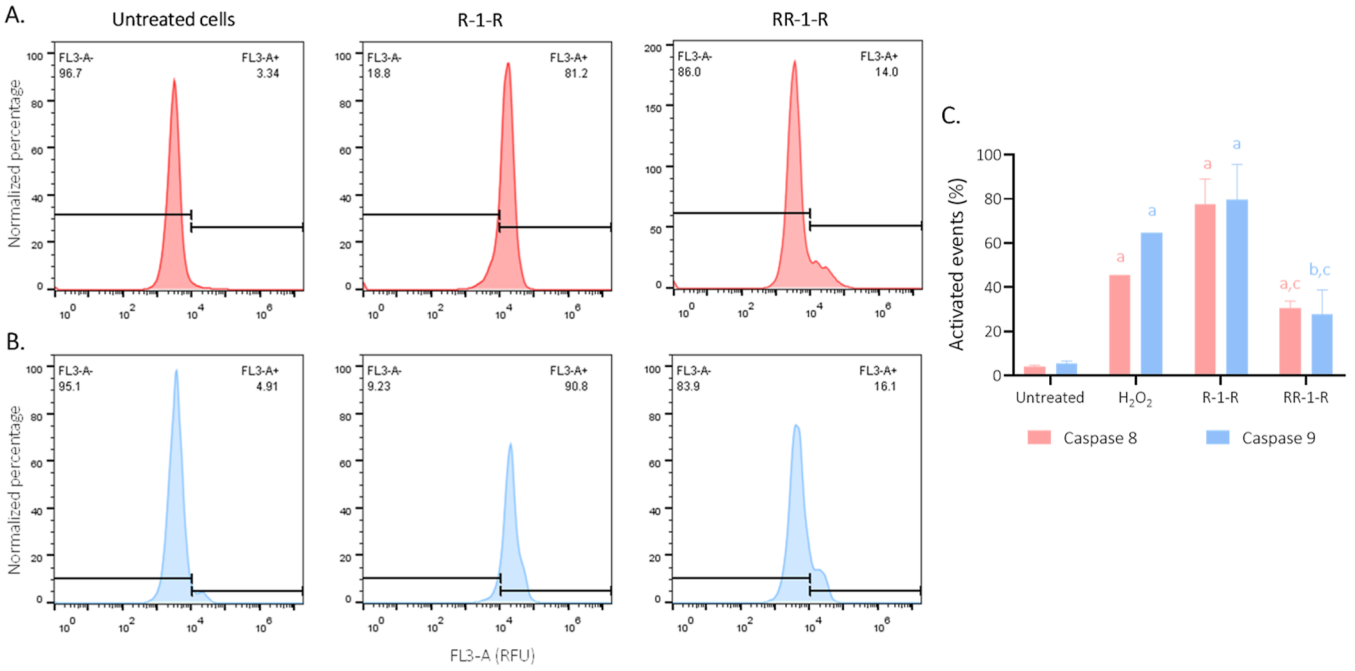

Figure 4. Flow cytometry assay of caspase activation in MCF-7 cells treated with the IC_{50} of the peptides R-1-R and RR-1-R. A. Representative histograms of the change in fluorescence with the evaluated treatments of activated caspase 8 and B. Caspase 9 C. Bar graph of the percentage of activation of caspases. ANOVA multiple comparisons of SIDAK. [a]Significant differences with cells without treatment, $p < 0.05$. [b]Significant differences with cells treated with H_2O_2, $p < 0.05$. [c]Significant differences with cells treated with R-1-R, $p < 0.0001$.

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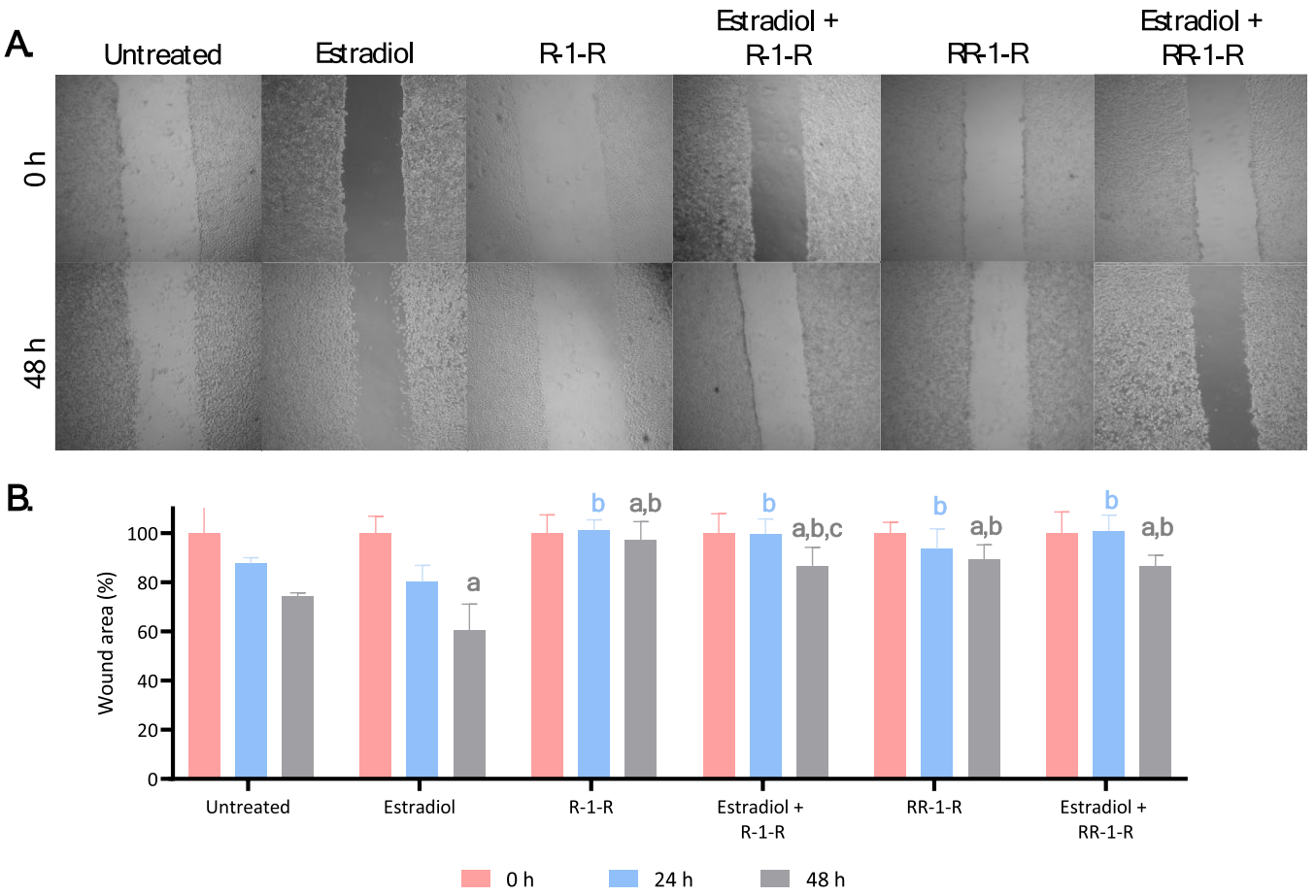

Figure 5. Wound-healing assay in MCF-7 cells treated with the IC_{50} of peptides R-1-R and RR-1-R A. Representative microphotographs of the wound area at 4× B. Bar graph of percentage of wound area after 24 and 48 h of treatment. (n = 12) ANOVA multiple comparisons of SIDAK. [a]Significant differences with untreated cells at the same time of evaluation, $p < 0.05$. [b]Significant differences with cells treated with estradiol at the same time of evaluation, $p < 0.005$. [c]Significant differences with cells treated with R-1-R at the same time of evaluation, $p < 0.05$.

RBC, and R-1 was hemolytic at concentrations over 12.5 μg mL^{-1}, with a maximum of 30% of hemolysis (Figure S3).

According to these results, peptides with one or two Arg residues at the N-terminal, RR-1-RR, RR-1-R, and R-1, with net charges of +6, +5, and +3, each showed an increased cytotoxic effect against at least one of the breast cancer cell lines and somewhat maintained a selective action with nonsignificant differential effects on fibroblast or MCF-12 cells (Table 3).

The *Galleria mellonella* model was chosen to further measure differences in the preliminary toxicity effect of the palindromic and modified peptide. Once again, no major changes were seen; after 10 days of continued monitoring, neither of them induced a survival rate below 50%, which is why lethal doses were not able to be determined. It is very interesting that at the highest dose of 800 μg mL^{-1}, the survival rate was 90% in both cases (Figure S4).

Type of Cell Death Induced. Since the modified peptide RR-1-R was the only one that exerted a significantly greater cytotoxic effect while maintaining the same selectivity as the palindromic peptide, it was chosen to preliminarily evaluate if this addition will induce a different type of cell death than that exhibited by the palindromic peptide R-1-R on the MCF-7 cell line. First, the significant amount of necrosis was evaluated through the measurement of internal calcium flux. Neither of the evaluated peptides induced a rapid release of it, suggesting no major necrotic events involved in the effect (Figure 3A). Also, a necrosis/apoptosis assay was performed. For the palindromic peptide, the results were similar to those previously reported, with most of the cell population at an apoptotic state in the same amount as that induced by modified RR-1-R peptide, but among these apoptotic cells, a significant difference was observed between the two treatments: while in the palindromic peptide R-1-R treatment most of the events presented were late apoptotic, in the modified peptide treatment, half of the cells exhibited late apoptosis and the other half exhibited early apoptosis.

To assess if the major apoptotic pathway activated by these two peptides was the same or differences in stimulation could account for the variations observed in the previous assay, activation of caspase 8 and 9 was calculated (Figure 4). For the palindromic peptide R-1-R, both the intrinsic and extrinsic pathways were triggered in nearly 80% of all events read at 2 h of treatment. On the contrary, the activation of these caspases when treated with RR-1-R was around 25%. This smaller activation may be the reason why most of the events were related to early apoptosis.

Effect on Migration. To estimate if these peptides could exert any other type of effect, a wound healing assay was carried out. 24 h after the wound was performed, about 20% of the wound area was reduced when cells were treated with estradiol or left untreated due to their migration to induce its

https://doi.org/10.1021/acsomega.2c07336
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closure, but for those cell cultures where R-1-R was added with or without estradiol, the wound area did not get any smaller (Figure 5). In a similar way, in cells treated with RR-1-R, the maximum area reduced was 5%, indicating that both peptides reduce their migration capability. By 48 h after being wounded, untreated cells had migrated, closing nearly 30% of the wound, while those treated with estradiol decreased the wound area by 40%. Those treated with the palindromic peptide only induced a 3% closure, and when estradiol was added, the area reduced by 14%. For those where RR-1-R was used, the wounded area was reduced by nearly 10% regardless of the use of estradiol, showing that cells under treatment with either of the peptides maintained a reduced migration, inhibiting the possibility of closing the wound.

DISCUSSION

AMPs are divided into groups according to their amino acid composition and structure. One of these groups consists of cationic peptides that lack cysteine residues; due to these characteristics, most of them are linear and most commonly have a disordered structure forming extending coils, are also rich in some amino acids such as Arg and Lys, and are fragments of larger proteins like casein, lysozyme, ovalbumin, and lactoferrin. According to their structure, peptides can be divided into four categories including linear α-helical peptides, β-sheet peptides, linear extension structures, and both α-helix and β-sheet peptides. However, short cationic peptides (6 to 12 residues) are linear and most commonly have a disordered structure forming extending coils. Some of them have exhibited an antimicrobial effect not only against Gram-positive and Gram-negative bacteria but also against viruses, fungi, and some types of cancer.[20] This direct cytotoxicity toward cancerous cells seems to be related to a major degree to their charge because cationic peptides can form electrostatic interactions with these cells' membrane, which have a net negative charge on the outer surface mainly due to the imbalance of lipids in the bilayer.[21] Also, the addition of Trp or another hydrophobic amino acid to this cationic sequence has improved the effect because of its ability to disrupt the cell membrane and reach intracellular targets.[22] Both approaches have been tested in the palindromic peptide R-1-R, whose configuration of alternation of basic and hydrophobic residues induces an advantageous effect over the lactoferricin minimal motif, inducing cytotoxicity against breast MCF-7, MDA-MB-231, MDA-MB-468, and colorectal CaCo-2 cancerous cell lines while exhibiting no or significantly less activity on HEK-293, MCF-12, CEMB, and primary culture fibroblasts.[6,15,23] Also, a replacement of Arg to Lys residues has been made in this palindromic sequence; this approach does not improve its antibacterial activity[24] or its cytotoxic effect.

Modifications of the length of active peptides have been shown to result in a major improvement of their antimicrobial activity. Furthermore, in Trp- and Arg-rich AMPs, it has been established that the overall antibacterial activity increases consistently with the length, being around 10 residues, which suggested for maximum effect.[25−27] Here, we assessed if this conclusion was the same for anticancer peptides (ACPs), and our results do not suggest such correlation since peptides RR-1-R and R-1-RR did not exert a similar cytotoxic effect, even though both have 10 residues, one more than the original peptide. In this case, the addition of Arg at the N-terminal induced a lower IC_{50} in both breast cancer cell lines, while when the addition was made at the C-terminal, the cytotoxicity induced was diminished when compared not only to its counterpart but also to the original peptide. Neither does the deletion of an amino acid creating two eight-residue peptides back up this hypothesis since similar results were obtained with the MCF-7 cell line, where deletion at the N-terminal reduced its cytotoxic effect while removal at the C-terminal potentiated it. These results defined new limits for ACPs since prior in silico studies of length showed that the optimal peptides were those with 21−30 amino acids,[28] and if we compare the effect on MCF-7 cells exerted by the full 25-residue LfcinB peptide at 200 μg mL^{-1} to the one obtained here by the palindromic or any of its modifications, the short peptides induce 20% more cell death, while for MDA-MB-231, it maintained a similar cell survival percentage[29] with 16 fewer residues on its sequence. This could be seen as an advantage since fewer residues correlate directly to a lower production cost and time, and also, they are less prone to inducing an immune response.[30]

The changes in the charge of the palindromic sequence were done in order to validate the assertion that there is a strong correlation between the antimicrobial activity and the net positive charge of peptides, establishing that a charge of +2 or more will induce bacterial death and +5 charge is ideal for reaching the maximum antibacterial effect, while a more positive peptide will cause loss of selectivity.[30−32] Regarding the application of these modifications to ACPs, there have been mixed results when it comes to peptides derived from LfcinB. Some reports have shown that an increase in net positive charge did not enhance antitumor activities,[33] while others have stated that in order to exert a high antitumor activity and selectivity, a +7 charge is needed.[34,35] Here, we suggest that net charge is not the only factor involved in higher anticancer activity as neither +5 peptides RR-1-R or R-1-RR nor +3 peptides 1-R and R-1 improved the activity in the same way. Those whose charge was imbalanced due to more Arg at the C-terminal lost their activity against both breast cancer cell lines, while their counterparts increased their cytotoxic effect, showing that the position alongside the peptide may have a greater influence than the charge itself.

Concerning selectivity, it is interesting that only in the 1 peptide when charge is diminished to +2 is the selectivity significantly reduced, inducing significantly greater cell death in normal mammary gland MCF-12 cells, primary culture fibroblasts, and red blood cells at the maximum concentration evaluated which may be due to the low cationicity and high rate of Trp residues since these results agree with the fact that cationic peptides with more than 60% of their residues hydrophobic lack selectivity.[18,36] The results for RR-1-RR and R-1-R are in accordance with the recent suggestion that an even spread of R residues is important for the cytotoxic effect due to an improved binding to the negative membrane.[25,37] Both peptides induced cytotoxicity in MCF-7 while maintaining their selectivity. It is remarkable to note that the peptide RR-1-R was the modification that induced a significantly greater cytotoxic effect against cancerous cell lines and did not affect the selectivity, and it may not directly correlate with the increase in charge or length of the peptide, as discussed before, but may be due to the fulfillment of the minimal action motif of LfcinB (RRWQWR) in its sequence, which may not only improve its anticancer activity but also maintain the selectivity observed in the palindromic sequence and minimal motif.

When we compare the type of cell death induced by RR-1-R peptide with that of the original palindrome R-1-R, both cause little necrosis and most of the events collected were due to

https://doi.org/10.1021/acsomega.2c07336
ACS Omega 2023, 8, 2712−2722

apoptotic cells being a significantly greater population on early apoptotic cells with RR-1-R treatment, which correlates with less activation of both caspases. It is intriguing to note that both peptides not only promote cell death but also diminish the capability of migration of the MCF-7 cell line even when estradiol is used. This may be a complementary way of action, where the migration and possible invasion of these cells to colonize other areas are significantly diminished. This result is particularly interesting since the opposite effects of LfcinB have been published in this area. Some have reported the opposite effect as complete human and bovine lactoferrin have been described as promotors of MCF-7 cell migration due to changes in protein concentration rather than to an specific via activation,[38] while others have shown that MCF-7 cells treated with iron-free or iron-saturated bovine lactoferrin for 24 h significantly reduced their migration capability.[39]

CONCLUDING REMARKS

Contrary to what has been reported for AMPs or in silico studies to design ACPs, length itself is not a characteristic that directly correlates with anticancer activity. Here, we have shown that increasing the residues in the palindromic peptide R-1-R does not necessarily improve its cytotoxic effect on MCF-7 or MDA-MB-231 breast cancer cell lines. In a similar way, an increase in the peptidés net charge does not in itself enhance the anticancer activity of the peptide. As has previously been stated for AMPs, the position where the charged residues are added is essential. In this particular case, peptides where the addition of Arg completes the minimum activity motif of LfcinB were those whose IC_{50} was diminished. Also, removal of the terminal Arg residues, generating a +2 peptide, correlates with the fact that the less the charge, the less the selectivity of the peptide, inducing cell death in the three normal cell types evaluated. Last, the RR-1-R peptide with Arg at the N-terminal exhibited increased anticancer activity while maintaining selectivity. This cytotoxic effect was related to early apoptotic events by activating the intrinsic and extrinsic pathways. Also, it reduced the migration of MCF-7 cells, proving that this and further modifications are promising molecules for developing in-depth studies of the action mechanism of short peptides derived from LfcinB. It is important to highlight that the main contribution of our research is that with the specific changes of 1 or 2 amino acids in the sequence, it is possible to modulate the anticancer activity as well as the selectivity, being a valuable tool to design and identify promising peptides.

MATERIALS AND METHODS

Reagents and Materials. Rink Amide Resin, Fmoc-Arg(Pbf)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-6-Ahx-OH, Fmoc-Lys(Fmoc)-OH, Triton-X, piperidine, *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), dicyclohexylcarbodiimide (DCC), 1-hydroxy-6-chloro-benzotriazole (6-Cl-HOBt), ninhydrin, potassium cyanide (KCN), ethanol, pyridine, phenol, trifluoroacetic acid (TFA), ethyl ether, triisopropylsilane (TIS), ethanedithiol (EDT), acetonitrile (ACN), methanol (MeOH), and phase extraction columns solid Supelco , Trypsin−EDTA solution, and pepsin 2500 units mg^{-1} were purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary cell culture of fibroblasts was obtained from the foreskin by the Laboratory of Cellular Physiology of the National University of Colombia, and the fibroblasts were cultured and frozen. Annexin V, Alexa Fluor 488 conjugate was purchased from Invitrogen (Eugene, Oregon). The RPMI 1640 culture medium and trypsin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bovine fetal serum, penicillin, and streptomycin were purchased from Gibco (Waltham, MA, USA). A calcium assay kit and a Fluorescein CaspGLOW Active Caspase-8 or Caspase-9 Staining Kit were obtained for BD Biosciences (Tor- reyana Rd., San Diego, CA 92121, USA) and Invitrogen Thermo Fisher Scientific (Waltham, Massachusetts, USA), respectively.

Solid-Phase Peptide Synthesis—Fmoc/*t*Bu. Palindromic and modified peptides were manually synthetized using the Fmoc-tBu strategy in accordance to Rodríguez et al.[40] In brief, rink amide resin (0.46 mEq g^{-1}) treated with DCM at room temperature (RT) for 2 h was used as a solid support. Elongation of the sequence was carried through sequential steps of (i) Fmoc group removal under basic conditions, treating the resin or resin−peptide with 2.5% v/v 4-methyl piperidine by 15 min (twice); (ii) Fmoc-amino acid/DCC/6-Cl-HOBt (1:1:1 meq and 5 excess with respect to resin substitution) were dissolved in DMF/DCM mixture (2:1 v/v); then two drops of Triton X-100 were added, and the reaction mixture was gently stirred for 15 min at RT. Then the reaction mixture was added to resin or resin−peptide and gently stirred for 2 h at RT. The peptide was separated from the resin treating the dried resin−peptide with TFA/water/TIS/EDT (92.5/2.5/2.5/2.5% v/v) and stirring for 8 h. Crude peptides were precipitated using diethyl ether at −20 °C and centrifuged at 2500 rpm for 5 min, and the supernatant was eliminated, and the diethyl ether remnant was eliminated by evaporation at RT.

RP-HPLC Characterization. Peptides (10 μL; 1 mg mL^{-1}) were analyzed on an Agilent 1260 HPLC (Omaha, NE, USA) with a UV−vis detector (210 nm) using a Chromolith C-18 monolithic column (50 × 4.5 mm). Solvent A: water with 0.05% TFA, solvent B: CAN with 0.05% TFA. Elution gradient program: 5/5/50/100/100/5/5 % B in 0/1/9/9.1/12/12.1/15 min. Flow rate: 2 mL min^{-1}.

Reverse-Phase Solid-Phase Extraction Purification. Peptides were purified by solid-phase extraction (SPE) chromatography in accordance with Insuasty Cepeda et al.[41] Briefly, 1 mL of crude peptide (25 mg dissolved in 1 mL of solvent A) was passed through SPE columns (SUPELCO LC-18 with 2.0 g resin) and then eluted with solutions containing different percentages of solvent B. Collected fractions were analyzed using RP-HPLC, and the fractions containing pure peptide were evaporated at RT, lyophilized, and analyzed using RP-HPLC.

Mass Spectrometry Analysis. 2 μL of pure peptide (10 μg mL^{-1}) was analyzed on a Bruker Impact II LC Q-TOF MS equipped with electrospray ionization (ESI) in positive mode. The chromatographic conditions were Intensity Solo C18 column (2.1 × 100 mm, 1.8 μm) (Bruker Daltonik), a temperature of 40 °C, and a flow rate of 0.250 mL min^{-1}. Water as solvent A water and ACN as solvent B, each containing 0.1% formic acid, were used as the mobile phase. The elution program was 5/5/95/95/5/5 % B at 0/1/11/13/13.1/15 min. ESI source conditions: end plate offset 500 V, capillary 4500 V, nebulizer 1.8 bar, dry gas nitrogen 8.0 L/min, and dry temperature 220 °C. Scan mode AutoMS/MS with a spectral range of 20−1000 m/z, a spectra rate of 2 Hz, and a collision energy of 5.0 eV were used.

https://doi.org/10.1021/acsomega.2c07336
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Cell Culture. MCF-7, MCF-12, MDA-MB-231, and fibroblast cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (SFB), with 1% penicillin−streptomycin. Media were filtered through a 0.22 μm membrane. Cell culture was maintained in an incubator (Sanyo serial no. 11030213) at 37 °C with 5% CO_2.

MTT Viability Assay. Cytotoxicity assays were performed as described by Barragán-Cárdenas et al.[15] In a 96-well plate, cells were seeded in a supplemented medium at a rate of 1×10^4 cells per well, adhesion was allowed for 12 h, and then the medium was replaced by an incomplete medium to allow cell syncing for 12 h. Cells were treated with peptide dissolved in the medium at concentrations of 200, 100, 50, 25, 12.5, and 6.25 μg mL^{-1}; after incubation for 2 h, 10 μL of MTT (5 mg mL^{-1}) per well was added and plates were incubated for 4 h. Crystals were dissolved in 100 μL of DMSO for 40 min, and absorbance was measured at 575 nm. DMSO as blank, the incomplete medium as a negative control, and H_2O_2 as a positive control were used.

Hemolytic Activity Assay. Hemolytic assays were performed as described by Solarte.[42] Briefly, 5 mL of blood in EDTA was centrifuged at 1000*g* for 15 min. The erythrocyte-rich fraction was resuspended in 10 mL of sterile saline solution and washed twice by centrifugation under the same conditions. Then 100 μL of erythrocytes (4% hematocrit) was mixed with peptide (concentrations ranging from 400 to 6.2 μg mL^{-1}) and incubated for 2 h at 37 °C. It was then centrifuged at 2500 rpm for 5 min, and the absorbance of the supernatant was measured at 450 nm. Two controls were used: Tween 20 as the positive control and saline solution 0.85% as the negative control.

Apoptosis/Necrosis Cytometry Assay. Cells were seeded and synchronized in 24-well plates at a rate of 4×10^4 cells per well and then were treated for 2 h with the IC_{50} of each peptide. Cells were harvested with trypsin, centrifuged at 2500 rpm for 10 min, washed with PBS, and suspended in 20 μL of staining buffer with the fluorochromes (annexin binding buffer containing 1 μL of 7AAD fluorochrome and 5 μL of Annexin V). Cells with the fluorochromes were incubated at 37 °C in the dark for 15 min and suspended in 80 μL of the staining buffer without fluorochromes for analysis on a BD Accuri C6 device. The positive control of necrosis was cells treated with EDTA 15 mM for 60 min, and the positive control of apoptosis was cells treated with actinomycin D at 15 μM for 24 h. Negative control: cells without treatment; compensation controls: (i) cells stained only with Annexin V and (ii) only with 7AAD; population control: unstained and untreated cells.

Calcium Intracellular Release. Cells were seeded and synchronized in 25 cm^2 flask plates at a rate of 1×10^5 cells per treatment and then were harvested, suspended in a mixture 1:1 of RPMI-1260 incomplete medium and staining buffer with 3 μL of calcium indicator, and incubated for an hour at 37 °C and 5% CO_2. 1×10^5 cells were analyzed for 6 min on a BD Accuri C6 device; a baseline fluorescence of 1 min was established; then the treatments were added, and the fluorescence intensity was monitored for another 5 min. For positive control, phorbol-myristate-acetate (PMA) 14 μM was used, and for negative control, the incomplete medium was used.

Caspase 8,9 Cytometry Assay. 1×10^6 cells were seeded, synchronized, and treated with IC_{50} of each peptide in a six-well plate for 2 h. After that, cells were harvested and transferred to a 1.5 mL centrifuge tube in 100 μL of the complete medium, and 1 μL of caspase 8 or 9 substrate was added and then incubated for an hour. Cells were centrifugated at 3000 rpm for 5 min and washed with wash buffer two times; the analysis was made on a BD Accuri C6 device.

Wound-Healing Assay. The cells were seeded and synchronized on a 12-well plate at a rate of 1.5×10^5 cells per well and then were treated with 0.1 mg mL^{-1} of mitomycin for 2 h. Vertical ruptures of the monolayer were made, and cells were treated with the IC_{50} of each peptide; every 12 h, microphotographs were taken for 48 h. All images were analyzed in ImageJ to measure the wound area.

In Vivo Toxicity Model on *G. mellonella*. The lower end of the larvae was cleaned with a swab and 70% hypochlorite, and subsequently, they were inoculated with 10 μL (last right leg) of the different concentrations of peptide (400, 600, and 800 μL); each treatment group was made up of 10 larvae and was performed in duplicate. The following were obtained as controls: 10 uncleaned larvae as the absolute control and 10 larvae that were cleaned and 10 larvae that were inoculated with 0.9% saline solution as an inoculation control. The larvae were then incubated at 37 °C and followed for 10 days. Viability data were analyzed using Kaplan Meyer curves.[43]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c07336.

Chromatographic profiles of the pure fraction of the peptides synthesized; high-resolution mass spectrum of the peptides synthesized; graph showing percentage of hemolysis with different concentrations of peptides synthesized; and graph showing in vivo toxicity effect of treatment with palindromic peptide R-1-R and peptide with incorporation of Arg residue in the RR-1-R on *G. mellonella* larvae (PDF)

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https://doi.org/10.1021/acsomega.2c07336
ACS Omega 2023, 8, 2712−2722

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was conducted with the financial support of COLCIENCIAS 807-2018, Project "Desarrollo de un medicamento contra el cáncer de mama basado en un péptido polivalente derivado de la LfcinB:Estudio de la fase preclínica (fase cero), caracterización fisicoquímica de un lote del fármaco para estudios preclínicos. Code 110180762973", contract RC no. 706-2018.

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