



Research paper

Crotalicidin and NA-CATH-ATRA-1-ATRA-1 peptide-induced membrane disruption in human breast cancer cells

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ABSTRACT

Cationic peptides offer a promising alternative for cancer treatment due to their ability to target cancer cells via standard membrane features, thereby overcoming intratumoral heterogeneity. This study investigates the cytotoxic activity and the membrane-disruptive effects of two snake venom-derived peptides, Crotalicidin (Ctn) and NA-CATH-ATRA-1-ATRA-1 (NA) in human breast cancer cells. Cell viability assays showed that both Ctn and NA significantly diminished the viability of MCF-7 and MDA-MB-231 cells, with NA showing greater potency, as indicated by lower IC₅₀ values of 13.4 μ M for MCF-7 and 6.4 μ M for MDA-MB-231. Microscopy and flow cytometry revealed size reduction and increased granularity in treated cells. Further analyses indicated that the peptides induced membrane permeabilization, as evidenced by significant propidium iodide uptake, without significantly altering mitochondrial membrane potential. Apoptosis markers such as cleaved caspase-9 and PARP, were not detected by western blot.

Additionally, LDH release and confocal microscopic analysis supported the findings of membrane disruption. Finally, infrared spectroscopy (FT-IR) on lipid extracts revealed peptide-membrane interactions, resulting in phase transitions consistent with membrane disruption. These findings highlight the potent cytotoxic effects of Ctn and NA on breast cancer cells and their potential as novel therapeutic agents.

1. Introduction

Breast cancer affects millions of women worldwide, and the number of new cases unfortunately increases every year. In 2022, the World Health Organization (WHO) reported that 2.3 million women were diagnosed with breast cancer, and 665,684 deaths occurred globally, making it the most prevalent cancer in the world [1]. The causes and origin of breast cancer are diverse and, at the same time, not fully understood [2]. Additionally, breast cancer is a highly heterogeneous

disease characterized by various molecular subtypes defined by the status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [3,4].

This molecular heterogeneity influences treatment responses, therapeutic failures, and disease outcomes [5,6]. Depending on the breast cancer subtypes and stages, there are several clinical options for treating the disease, such as surgery, chemotherapy, targeted therapy, immunotherapy, and radiation therapy [7,8]. Unfortunately, despite the current therapeutic options, many cases remain clinically unmanageable

Abbreviations: Ctn, crotalicidin; NA, NA-CATH-ATRA-1-ATRA-1; FT-IR, infrared spectroscopy; WHO, World Health Organization; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; CPs, cationic peptides; C. durissus, *Crotalus durissus*; HPLC, High performance liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MTT, 3-(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FSC, forward scatter; SSC, side scatter; FSC-A, forward scatter area; FSC-H, forward scatter height; DiOC₆(3), 3,3'-dihexyloxycarbocyanine iodide; PBS, phosphate-buffered saline; PI, propidium iodide; MFI, mean fluorescence intensity; SDS-PAGE, SDS-polyacrylamide gels; PVDF, polyvinylidene difluoride; LDH, lactate dehydrogenase; PS, phosphatidylserine.

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and deadly due to acquired or intrinsic resistance to treatment [9,10]. Therefore, there is an imperious need to evaluate new drugs against breast cancer. Among these potential therapeutic agents, cationic peptides (CPs) have arisen indirectly as an attractive alternative for cancer treatment. CPs are part of the chemical armory of the non-specific immune systems of several organisms. The most recognized activity has been associated with antimicrobial action. However, CPs have shown a broad spectrum of activities as antibiotics, antiviral, antiprotozoal, and in the last decade as promising anti-cancer agents [11,12]. The most attractive characteristic of CPs as potential agents in breast cancer treatment lies in their fast biological activity targeted at membranes, bypassing “classical” mechanisms of drug resistance [13]. The most widely accepted CPs mechanism of action is established on a non-specific interaction between the positively charged amino acids of the peptide and the cell membrane’s negatively charged groups. This interaction disrupts the cell membrane, causing the leakage of cytoplasmic content and ultimately leading to cell death [14]. However, CPs might penetrate the membrane and interact with intracellular targets, such as mitochondria [15]. Specifically, CPs with antitumor activity tend selectively towards malignant cells compared to non-tumorous ones. This selectivity is linked to their capacity to bind to the negatively charged phosphatidylserine (PS). This phospholipid is predominantly located in the outer leaflet of plasma membranes in cancer cells [16].

One of the most successful peptides is LTX-315 (Oncopore), a synthetic cationic sequence derived from human lactoferrin that has demonstrated activity against soft tissue sarcoma, basal cell carcinoma, advanced melanoma, and breast cancer cell lines [17]. LTX-315 is recognized for being a pioneering oncolytic peptide in human clinical trials [18,19]. LTX-315 also represents an innovative approach to cancer immunotherapy; it has been demonstrated that intratumoral injection of the peptide induces complete tumor regression in untreated tumor areas [20]. Currently, LTX-315 is under a phase II study to estimate the efficacy and safety of the intratumoral peptide injection combined with pembrolizumab, a humanized antibody used in cancer immunotherapy for the treatment of melanoma [21].

Snake venoms are natural sources rich in a cocktail of biologically active molecules composed mainly of peptides or proteins [22]. Among these components, numerous families of peptides have been identified that present different activities at the physiological level, such as antimicrobial, antihypertensive, analgesic, and antiparasitic, among others [23]. From the venom of the species *Crotalus durissus* (*C. durissus*) known as rattlesnake, Crotalicidin (Ctn) has been isolated [24–26] and the peptide NA-CATH-ATRA-1-ATRA-1 (hereafter referred to as NA), is a derivative of the peptide NA-CATH, isolated from the species *Naja atra* or Chinese cobra [27–29]. Although the antimicrobial activity of these peptides has been reported against some microorganisms, such as *Staphylococcus aureus* [26,29,30], the antitumor effect has only been demonstrated for Ctn, but not in breast tumor cells [25]. In this research, we evaluated and compared the cytotoxic activity of Ctn and NA in MCF-7 and MDA-MB-231 human breast cancer cells, as well as spontaneously immortalized human keratinocytes (HaCaT), with that of LTX-315. The consequences of the peptide treatments on cell membrane integrity, mitochondrial function, and cell cycle distribution were determined with flow cytometry. We evaluated the disruption of the cell membranes by LDH release and confocal microscopy. Finally, we investigated the direct interaction between synthetic peptides and membrane lipid extracts of HaCaT, MCF-7, and MDA-MB-231 cells using infrared spectroscopy (FT-IR).

2. Material and methods

2.1. Peptide synthesis

Crotalicidin (Ctn, KRFFKFFKVKSVKKRLKKIFKKPMVIGVTIPF, Lot. U037QFC180-1/PE6093), NA-CATH-ATRA-1-ATRA-1 (NA,

KRFFKFFKVKLSVKKRFFKFFKLVIGVTIPF, Lot. U037QFC180-11/PE6100), and LTX-315 (KKWWKKWDipK–NH₂, Dip: Diphenylalanine, Lot. U037QFC180-14/PE6102) were obtained according to the reported sequence by the solid-phase method and purchased from GenScript (Piscataway Township, NJ, USA). Trifluoroacetic acid removal was performed. The purity and molecular weights of the peptides were determined by HPLC (higher than 95 %) and MALDI-TOF mass spectrometry, respectively.

2.2. Cell culture

Human breast adenocarcinoma (MCF-7, ATCC HTB-22), triple-negative human breast cancer (MDA-MB-231, ATCC CRM-HTB-26), and non-tumoral human keratinocyte cells (HaCaT, CLS 300493) were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 5 % fetal bovine serum (FBS), and 100 µg/ml of penicillin and streptomycin. The cells were cultured and stored in a humidified incubator at 37 °C supplied with 5 % CO₂/95 % air. Cell cultures were periodically analyzed under a microscope to follow morphology, adherence, and exponential growth. Cells were trypsinized, pelleted, and analyzed using different tests to evaluate the effect after peptide treatments. All experiments were performed at least three times per treatment group.

2.3. Cell viability assay

The effect of the Ctn, NA, and LTX-315 on cell viability was determined using the colorimetric 3-(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay (Sigma-Aldrich, St. Louis, MO, USA M2128). For the experiments, 1×10^4 cells were seeded in 96-well plates, cultured, and stored under the culture conditions described previously. After 24 h to ensure cell adhesion and exponential growth, peptide treatments were applied at different concentrations as indicated and incubated for 24 h. Following that period, the medium was removed, and 100 µl of fresh medium with MTT (0.5 mg/ml) was directly added to the cells and incubated for 2 h at 37 °C in the dark. Finally, MTT was removed, and the formazan crystal was dissolved by adding 100 µl of acidic isopropanol (80 mM HCl and 10 % of Triton™ X-100 (v/v) in isopropanol). Using a MultiScan Go (Thermo Fischer Scientific, Waltham, MS, USA), the MTT formazan absorption was recorded at 570 nm. The results were reported as the percentage of cell viability where the OD measure from untreated cells was considered 100 % of cell viability. The absolute IC₅₀ was obtained using GraphPad Prism software 8.0.1. The Selectivity index (SX) was calculated following Eq. (1).

$$SX = \frac{IC_{50} \text{ non-tumoral cells}}{IC_{50} \text{ tumoral cells}} \times 100 \quad (1)$$

2.4. Morphological parameters analysis

These experiments were performed to follow the morphological effects induced by the peptide incubation on the breast cancer cells. For these experiments, 1×10^5 adherent MCF-7 and MDA-MB-231 were seeded into a 24-well plate and cultured under normal conditions. After 24 h, peptides were added at different concentrations (3.125 to 50 µM) and incubated for 6 h. Afterward, the cells were washed and photographed using a ZOE Cell Imager microscopy (Bio-Rad, Hercules, CA, USA). Furthermore, forward scatter (FSC) and the side scatter (SSC) parameters were used to assess the relative size and granularity of the cells, respectively. Flow cytometry was used to acquire 1×10^4 events per sample. Values were reported as scattering signal intensities.

2.5. Cell cycle analysis

The cell cycle distribution was evaluated using flow cytometry. This study cultured 1×10^5 cells in 24-well plates for 24 h. Subsequently, the

cells were treated with several subtoxic peptide concentrations (3.125 to 50 μ M) over 6 h. After this exposure, the cells underwent two sequential washes with PBS, enzymatic detachment through trypsinization, and fixation using 70 % cold ethanol. Next, the permeabilized cells were treated with 100 μ g/ml of RNase (Sigma, St. Louis, MO, USA, R5000) and stained with 100 μ g/ml of propidium iodide (Sigma, P4170) for 20 min. Fluorescence data were collected using the BD LSRFortessa™ Cell Analyzer (BioSciences, Franklin Lakes, NJ, USA) after removing doublets by plotting forward scatter area (FSC-A) versus forward scatter height (FSC-H). Subsequently, the DNA content within each cell cycle phase was analyzed using FlowJo v10.6.2 (Franklin Lakes, NJ, USA). The cell cycle results were analyzed based on the total DNA cell content. It was divided into two distinct gates: hypodiploid sub-G0/G1 and diploid /tetraploid cells (characterized by the absence of DNA fragmentation). The latter gate was used to investigate cell cycle distribution. All reported data were derived from a minimum of three independent experiments conducted for each treatment group, ensuring the robustness and reliability of the results.

2.6. Cytoplasmic membrane permeabilization and mitochondrial functionality

Experiments were performed to determine whether Ctn, NA, and LTX-315 could affect the cytoplasmic membrane integrity and 3,3'-diethylxycarbocyanine iodide (DiOC₆(3)) uptake changes of MCF-7 and MDA-MB-231 cancer cells. For these experiments, 1×10^5 cells were grown into a 24-well plate for 24 h and then incubated with different peptide concentrations for 6 h (3.125 to 50 μ M). After that, phosphate-buffered saline (PBS) was used to wash cells twice, then cells were trypsinized, pelleted, and centrifuged at 500 $\times$ g for 5 min at 20 °C, and the supernatant was removed, leaving about 300 μ l to resuspend the cells by vortexing. Then, cells were stained with 6 μ M propidium iodide (PI, Sigma, St. Louis, MO, USA, P4170) and DiOC₆(3) at 700 nM (Molecular Probes, Eugene, OR, USA, D273), and stored at room temperature for 20 min protected from light. After staining, cells were washed with 500 μ l of filtered FACS sheath fluid, followed by centrifugation at 500 $\times$ g for 5 min at 20 °C. Afterward, cells were analyzed by BD LSRFortessa flow cytometer (BioSciences, Franklin Lakes, NJ, USA) with FACS DIVA software. Aggregates were excluded, and the reading threshold was defined with non-stained cells. For each sample, at least 1×10^4 events were read. Each chart point represents the average of four biological replicates processed independently. For analysis, the mean fluorescence intensity (MFI) of DiOC₆(3) and the percentage of PI-positive cells were evaluated utilizing the FlowJo v10.6.2 software.

2.7. Apoptosis protein analysis by Western blotting

MDA-MB-231 cell line was plated 5×10^5 cells per well in a 6-well plate. Then, cells were treated with different peptide concentrations (3.125 to 50 μ M) for 6 h. After that time, cells were rinsed three times with PBS and lysed using M-PER buffer (Thermo Scientific) accompanied with protease and phosphatase inhibitors (ThermoFisher Scientific, Waltham, MA, USA). Protein lysates were mixed with a 5 $\times$ sample buffer (50 % glycerol, 10 % SDS, 0.5M DTT, and 250mM Tris pH 6.8) and boiled for 5 min at 98 °C. Samples and ProSieve™ Protein Ladder (Lonza, #50550, Basel, Switzerland) were separated on SDS-polyacrylamide gels (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). Membranes were subsequently blocked for 60 min in 5 % milk at room temperature in Tris-buffered saline with 0.1 % Tween-20 (TBS-T; 19mM Tris base, 137mM NaCl, 2.7mM KCl and 0.1 % Tween-20) and cultured with primary antibodies at 4 °C overnight, followed by three washes with TBS-T (5 min each). Immunodetection was performed using antibodies directed against cleaved PARP1 (1:1000; rabbit; Cell Signaling Technologies, cat. no. 5625, 89 kDa), cleaved caspase-9 (1:1000; rabbit; Cell Signaling Technologies, cat. no. 9501, 37kDa), and beta-Actin

(1:10,000, MP Biochemicals, #69100, 42 kDa). Finally, secondary antibodies (HRP-conjugated, 1:1500, DAKO, Glostrup, Denmark) were incubated at room temperature for 60 min, and to visualize bands, was employed Lumi-Light substrate (Roche, Basel, Switzerland) was employed to visualize bands using the ChemiDoc Imaging System (BioRad, Hercules, CA, USA).

2.8. Cell membrane disruption analysis

2.8.1. Release of lactate dehydrogenase (LDH)

Following the manufacturer's instructions, the cell membrane disruption induced by cationic peptides was evaluated using the LDH-Glo™ Cytotoxicity Assay (Promega Corporation, Catalog No. J2380). 1×10^4 Cells were seeded in a 96-well plate and treated with Ctn and NA at various concentrations (3.125 to 50 μ M) for 6 h. To establish 100 % LDH release, 10 μ l of 10 % Triton X-100 in PBS was added to designated wells. Following treatment, supernatants were collected and frozen in LDH storage buffer at a 1:20 dilution. Before analysis, the samples were thawed, and 50 μ l of each diluted sample was combined with 50 μ l of LDH detection reagent in an opaque 96-well plate. Luminescence was recorded using a CLARIOstar multimode plate reader (BMG LABTECH, Ortenberg, Germany), and the results were normalized to the 100 % release control. Data were expressed as a percentage of total LDH release, representing the extent of cell membrane disruption.

2.8.2. Confocal microscopy

The cell membrane disruption was assessed using the confocal CellDiscoverer 7 (CD7) system. MDA-MB-231 and MCF-7 cells were seeded in a 24-well plate at a density of 1×10^5 cells per well and allowed to adhere overnight. The following day, cells were treated with increasing concentrations of Ctn and NA peptides for 6 h. After treatment, the medium was removed, and a staining solution was added. The staining solution consisted of Hoechst 33342 (4.4 μ M; Invitrogen, C#H3570), Calcein-AM (1 μ M; BioLegend, C#425201), and Propidium Iodide (PI, 5.0 μ g/mL; Sigma-Aldrich, P4864), prepared in culture medium. Cells were incubated with the staining solution at 37 °C and 5 % CO₂ for 1 h. Confocal imaging was performed under controlled conditions of 37 °C and 5 % CO₂ using a 20 $\times$ magnification lens. Hoechst 33342 was excited with a 405 nm laser, and emission was collected between 410 and 440 nm. Calcein-AM was excited with a 488 nm laser, and emission was detected between 499 and 529 nm. PI was excited with a 561 nm laser, and emission was collected between 580 and 630 nm. Image acquisition and analysis were performed using Zeiss Zen Blue software.

2.9. Direct interaction of peptides with membrane phospholipids

2.9.1. Total lipid extract

Cell lines MCF-7, MDA-MB-231, and HaCaT were properly grown, trypsinized, and pelleted. Then, using 2 ml of filtered and deionized water, cells were washed and centrifuged at 6000 rpm at 4 °C for 15 min in plastic tubes. After discarding the supernatant, the pellet was freeze-dried at -60 °C, pressure < 260 μ bar (SP Scientific, NY, USA). The Bligh and Dyer lipid extraction method suitable for cell samples or tissues was used to obtain the total lipid extract [31]. Lyophilized cells were resuspended in 5 ml of deionized water to guarantee homogenization vortexed. The suspension was transferred into a clean glass separatory funnel for 96 ml of solvent mixture chloroform: methanol: water (1:2:0.8 v/v/v, including the cell suspensions. After the solvent addition, the system was shaken for 30 s. This latter process was often repeated for 18 h. To obtain a biomass-solvent mixture, chloroform was added at a final ratio of chloroform: methanol: water (1:1:0.4 v/v/v). Into a 100 ml flask bottle, the chloroform phase was transferred for its concentration and washed 3 times with 0.9 % NaCl (w/v). After recovering the organic phase, the solvent was evaporated using a nitrogen stream and vacuum inside a 1.5 ml glass vial. Before the analysis, the extracts were stored at

−20 °C [32].

2.9.2. Phase transition measurements by Fourier transform infrared spectroscopy (FT-IR)

Phase transitions were followed using a BioATR II cell from a Tensor II spectrometer (Bruker Optics, Ettlingen, Germany) with an MCT detector and a circulating water bath Huber Ministat 125 (Huber, Offenburg, Germany) to control the temperature (± 0.1 °C). The experiments were performed with 120 scans per measured temperature and a spectral resolution of 4 cm^{-1} . The first step for the phase transition measurements was to register the background between 5 and 45 °C with a heating rate of 1 °C/min and an equilibration period of 120 s between each measurement. After finalizing the background, the crystal of the BioATR II cell was coated by adding 0.3 mg of the total lipid extract in chloroform. The solvent was evaporated, and lipid film formed; 20 μl of the buffer (10 mM HEPES at pH 7.4) was used to hydrate the film for 10 min at 37 °C. Then, samples were analyzed using the same temperature range set as the background. For the peptides, during hydration, 1, 5, and 10 M% of the peptides were added in the same buffer.

The OPUS 3D 8.8.4 software (Bruker Optics, Ettlingen, Germany) processed the data by automatically removing the background from each recorded sample. The methylene group vibrations (2970 to 2820 cm^{-1}) were used to investigate the main transition temperature (T_m). The symmetric extension's frequency range (2850 to 2853 cm^{-1}) was extracted from the spectra and then baseline corrected using the 20 % sensitivity Rubber band method. Using the peak selection tool, the greatest wavenumber for the symmetric extension of each temperature was found. To calculate T_m , the experimental sigmoidal curve was fitted into a Boltzmann function using Levenberg Marquardt's iteration algorithm. $\nu_s\text{CH}_2$ were then plotted as wavenumber as a function of temperature using Origin Pro 8.0 software (Origin Lab Corporation, USA) [33].

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0.1 software (GraphPad, California, USA). One-way and two-way analysis of variance (ANOVA) was performed with post hoc comparisons via Fisher's least significant difference (LSD) test. The error bars represent the standard errors of the mean (SEMs). Results were considered significant at a level of 0.05 (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3. Results

3.1. Effect of Ctn and NA peptides on the cell viability of human breast cancer cells

MTT cytotoxic experiments determined the *in vitro* dose dependent effect of the peptides Ctn and NA on the human breast cancer cell lines MCF-7, MDA-MB-231 and the immortalized human keratinocyte cell line HaCaT. LTX-315 was selected as the control peptide. The results of the cytotoxic activity of Ctn, NA, and LTX-315 in the different cell lines are presented in Fig. 1. The analysis of the results showed that the viability of both cancer cell lines was significantly reduced with increasing concentrations of the three peptides. The predicted half-maximal inhibitory concentrations (IC_{50}) of the Ctn, NA, and LTX-315 are summarized in Table 1. The most significant effect on the viability of MCF-7 and MDA-MB-231 cells was observed with NA with IC_{50} values of 13.4 μM for MCF-7 cells and 6.4 μM for MDA-MB-231 cells.

Additionally, MDA-MB-231 cells were more affected by the peptides compared to MCF-7 cells, as indicated by the IC_{50} values of Ctn and NA, which were 2.8 and 2.1 times lower for MDA-MB-231, respectively. Notably, the heightened sensitivity of MDA-MB-231 cells to each of the three peptides resulted in a cytotoxicity ratio between HaCaT and MDA-MB-231 cells exceeding 100 (SX). However, in the case of MCF-7 cells, Ctn and NA exhibit similar or even more toxicity towards non-tumoral HaCaT cells, resulting in an SX of around 100 and <100, respectively

Table 1

Half-maximal inhibitory concentrations (IC_{50}) of the peptides Ctn, NA, and LTX-315 on non-tumoral HaCaT cells, and breast cancer cells MCF-7 and MDA-MB-231. * The selectivity index (SX) was calculated for MCF-7 and MDA-MB-231 cells.

Cell line	IC_{50} (μM)			Selectivity Index (SX)*		
	Ctn	NA	LTX-315	Ctn	NA	LTX-315
HaCaT	61.9	10.4	58.5	–	–	–
MCF-7	58.9	13.4	40.3	105.1	77.3	145.0
MDA-MB-231	21.3	6.4	40.8	290.3	161.2	143.3

Cells were treated for 24 h with peptides, after which IC_{50} values, e.g., the concentration at which cells had 50 % viability compared to the untreated cells (0 μM), were calculated from the resulting viability curve. Data represents mean $\pm$ SEM of three independent experiments performed in triplicate. *SX value ≥ 100 represents that the cytotoxicity effect is more selective towards breast cancer cells.

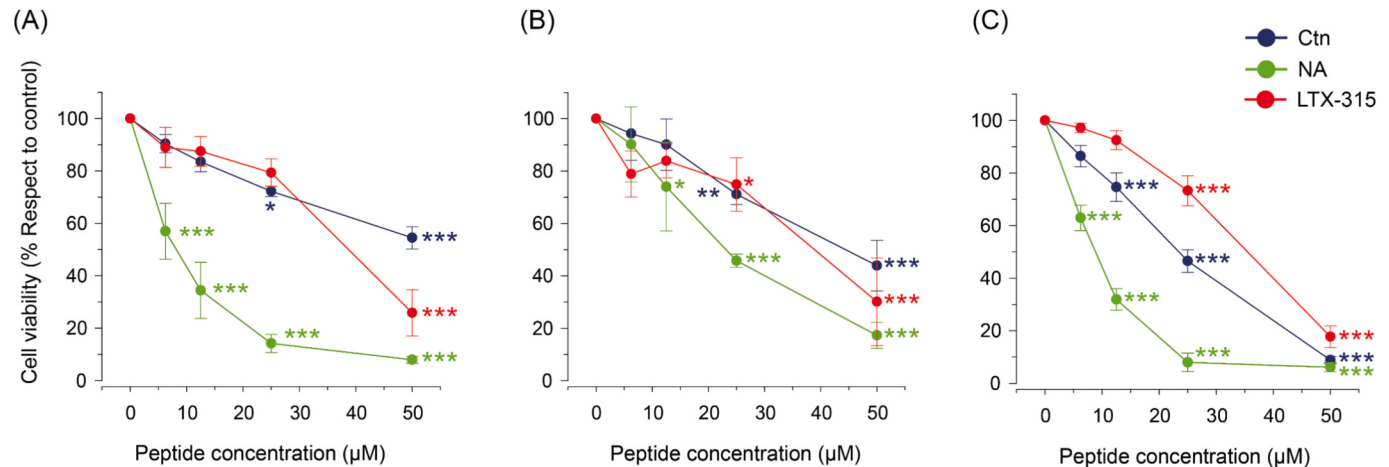


Fig. 1. Cytotoxic effect of Ctn (—●—), NA (—●—), and LTX-315 (—●—) in (A) HaCaT, (B) MCF-7, and (C) MDA-MB-231. Cells were incubated with different peptide concentrations (6.25, 12.5, 25, and 50 μM) for 24 h. The data shown represents the mean $\pm$ standard error of the mean (SEM) of three independent experiments performed in triplicate. Two-way ANOVA and post hoc test Fisher's LSD presented the difference concerning untreated cells (0 μM), where * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

(Table 1). Based on the MTT results, concentrations for each peptide were selected within the range of observed IC_{50} values for MCF-7 and MDA-MB-231 cells and used for further experiments (Table 2).

3.2. Morphological assessment of Ctn and NA-treated breast cancer cells

After a 6-hour treatment with the peptides, morphological changes, characterized by a decrease in size and an increase in granularity, were observed in non-tumoral HaCaT cells and MCF-7 and MDA-MB-231 breast cancer cells. These changes were related to increasing peptide concentrations (Figs. 2A, C, and S1). Similar morphological changes were induced by LTX-315 in HaCaT, MCF-7, and MDA-MB-231 cells (Figs. S1 and S2). The morphological changes were quantified using flow cytometry based on forward scatter/side scatter (FSC/SSC) parameters (Fig. 2B and D). The changes in FCS/SSC, flow cytometry analyses, align with the observed morphological changes for Ctn and NA in both cell lines, including cell shrinkage and loss of membrane integrity. Significant increases in SSC ($p < 0.05$) for cells treated with NA at 6.25 and 12.5 μ M in both tumor cells support the enhanced cytotoxic potency of NA over Ctn against MCF-7 and MDA-MB-231 cells, as demonstrated by the MTT assay. LTX-315-induced SSC changes occur at a similar concentration as observed for Ctn (25 and 50 μ M) (Fig. 2).

3.3. Effect of Ctn and NA on the sub-G1 population of MCF-7 and MDA-MB-231 cells

Next, we investigated whether the peptides had an effect on the cell cycle distribution or induced a sub-G1 population, which reflects cells that have lost nuclear DNA. The cell cycle distribution of MCF-7 and MDA-MB-231 cells after exposure to Ctn, NA, and LTX-315 peptides after 6 h are presented in Fig. 3. There was a notable concentration-dependent increase in the sub-G1 population, reaching approximately 96 % in MCF-7 cells and 72 % in MDA-MB-231 cells. Notably, Ctn and NA increased the sub-G1 population even at low concentrations, whereas LTX-315 showed a comparable increase only at high concentrations (Fig. 3A and C). These findings align with Ctn, and NA's superior cytotoxic activity compared to LTX-315. While the overall distribution of cells in the G0/G1, S, and G2 phases remained relatively unchanged at lower peptide concentrations, at higher concentrations cell populations shifted to the left with a marked increase in the sub-G1 peak, suggesting extensive DNA fragmentation. Morphological results from Fig. 2 supported this interpretation, as cells exposed to higher peptide concentrations showed clear signs of membrane disruption, consistent with peptide-induced lysis rather than mitotic arrest. These findings suggest that Ctn and NA cytotoxic activity is linked to the induction of a sub-G1 population, which can reflect either apoptosis or necrosis [34–36], but it is not strongly linked to targeting of cells in a specific cell cycle phase.

3.4. Effect of Ctn and NA on mitochondrial functionality and plasma membrane integrity

The membrane-disrupting activity of cationic peptides (CPs) is well-documented as a mechanism underlying their biological effects [37–39]. However, alternative mechanisms for their anti-tumor activity have also been proposed, including the peptides' ability to enter the cytoplasm

Table 2

Doses peptide concentrations selected for MCF-7 and MDA-MB-231 cell treatments.

Peptide doses*	Peptide concentration (μ M)		
	Ctn	NA	LTX-315
Low	12.5	3.125	12.5
Medium	25	6.25	25
High	50	12.5	50

and interfere with internal targets, such as those in mitochondria [15]. Therefore, we evaluated whether a 6 h treatment with the peptides (Table 2) induced mitochondria-dependent apoptosis in MCF-7 and MDA-MB-231 cells by monitoring the loss of mitochondrial membrane potential ($\Delta\Psi$ m) [40,41]. Propidium iodide (PI) was used as a control to determine if cells have lost membrane integrity. The untreated cancer cells (0 μ M) showed an evident accumulation of the fluorescent probe DiOC₆(3) but not of PI, as depicted in Fig. 4. No substantial reduction in the median fluorescence intensity (MFI) of DiOC₆(3) was observed in MCF-7 cells at any peptide concentration and even a slight increase in MFI of DiOC₆(3) in MDA-MB-231 cells after peptide treatment (Fig. 4B and D). These findings indicate that the peptides did not trigger a loss in mitochondrial membrane potential. In addition, flow cytometry analysis of propidium iodide (PI) positive cells showed that Ctn and NA peptides induced membrane leakage in MCF-7 and MDA-MB-231 cells, with over 30 % of cells affected at medium doses and over 70 % at higher doses (Fig. 4B and D). Similar effects were observed with LTX-315 (Fig. 4). The absence of cleaved caspase-9, a read-out for activation of the mitochondrial apoptotic pathway, and cleaved PARP1, a downstream marker of apoptosis, after 6 h of peptide treatment confirmed the flow cytometry results (Fig. S3).

Taken together, these results show that the mechanism of action of Ctn, NA, and LTX-315 does not involve the induction of apoptosis but is the loss of cell membrane integrity.

3.5. Cell membrane disruption analysis

To determine whether the activity of Ctn and NA is linked to peptide-membrane interactions, we measured LDH release into the culture medium as an indicator of plasma membrane damage caused by the peptides' membrane destabilizing effects. The results demonstrate that Ctn and NA produced a dose-dependent increase in LDH release in MCF-7 and MDA-MB-231 cells after 6 h of treatment (Fig. 5A). NA triggered the highest degree of LDH release for both cell lines and MDA-MB-231 cells showed a greater sensitivity to Ctn and NA peptides than MCF-7 cells. Consistent with the LDH cytotoxicity assay, confocal microscopy analysis confirmed the membrane-damaging effects of NA in MCF-7 and MDA-MB-231 cells (Fig. 5B and C). A concentration-dependent increase in PI-positive cells was observed, indicating enhanced membrane permeability and cell death at higher peptide concentrations. Furthermore, PI and Hoechst 33342 staining of treated MCF-7 and MDA-MB-231 cells demonstrated that nuclear condensation, which is another characteristic of apoptotic cells, was not present. The staining intensity of Hoechst 33342 in PI-positive cells is lower as compared to that in PI-negative cells (Fig. 5B and C). It is tempting to speculate that PI-positive cells have already lost part of the nuclear DNA. Figs. S4 and S5 show confocal microscopy images of MCF-7 and MDA-MB-231 cells treated with Ctn and NA across the full range of concentrations employed.

In summary, this observation aligns with our above-mentioned findings, indicating that the peptides induce cell death through a non-apoptotic mechanism, most likely membrane disruption.

3.6. Study of the lipid-peptide affinity by FT-IR

Phase transition measurements were performed to understand if the interaction with lipids present in the cell membranes of HaCaT, MCF-7, and MDA-MB-231 mediates the activity of the peptides. Through the symmetric stretching band of the methylene groups (ν_sCH_2) from the acyl chains of the phospholipids, infrared spectroscopy can be used to monitor the thermotropic behavior of membrane lipids. Temperature dependence of the wavenumber values at the peak positions of the total lipid extract of the three cell lines incubated in the presence of different concentrations of the peptides is displayed in Fig. 6. The results showed that total lipid extract presents a thermotropic behavior characteristic of the lipid systems that contain cholesterol compared to lipid systems in the absence of cholesterol [32].

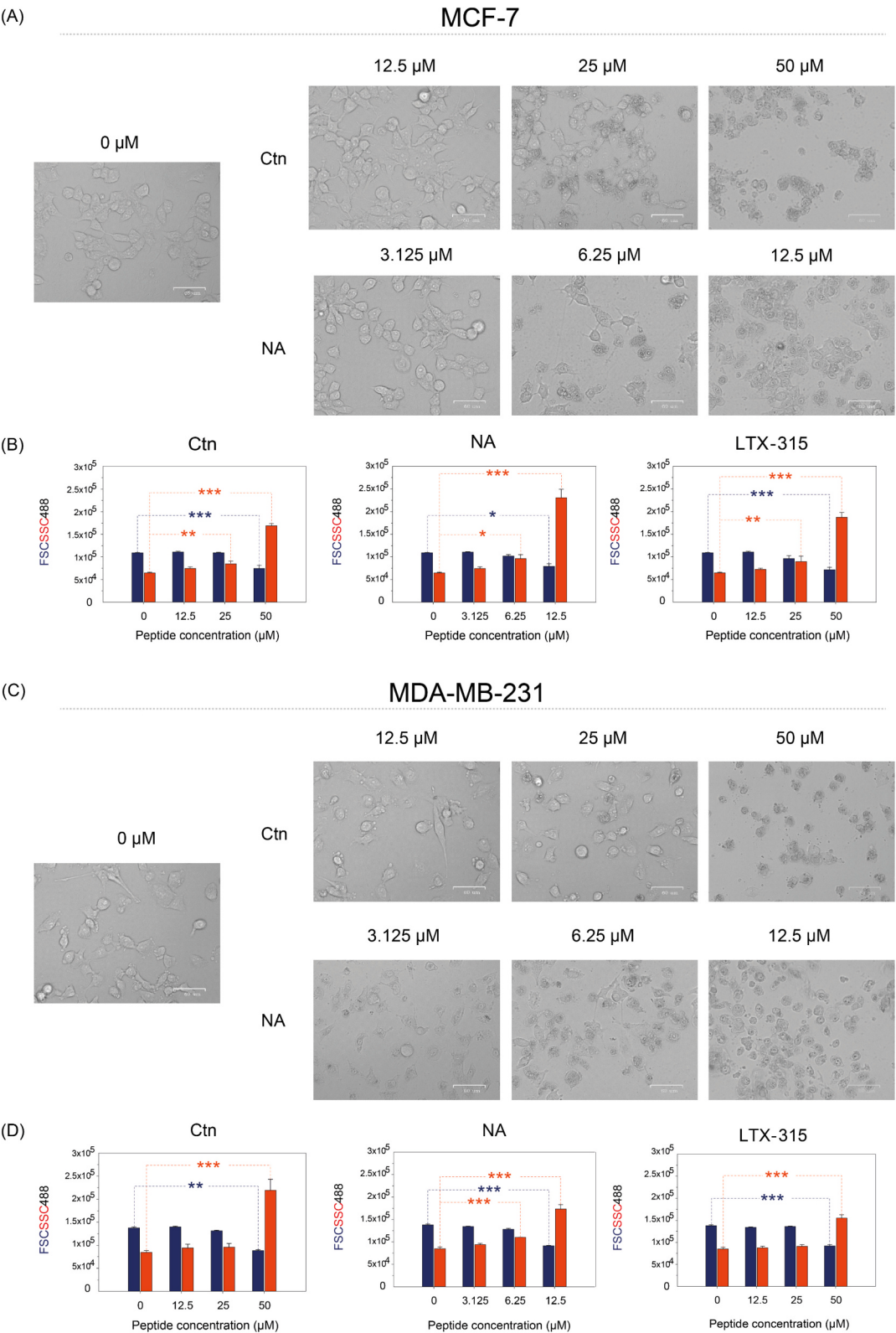


Fig. 2. Evaluation of morphological changes of MCF-7 and MDA-MB-231 cells after 6 h of treatment with different concentrations of Ctn, NA, and LTX-315. Direct observation by ZOE Cell imager microscopy of (A) MCF-7 (C) MDA-MB-231 cells evaluated by bright-field. Representative images at 10 $\times$ magnification of untreated (0 μ M) and cells treated with Ctn and NA for 6 h. The mean of the signal intensities detected by flow cytometry's forward scatter (FSC) parameter (—), and side scatter (SSC) parameter (—) corresponds to the measurement of the cell size and cell granularity (B) MCF-7 and (D) MDA-MB-231, respectively. The mean $\pm$ SEM of three independent experiments is used to express data. The differences compared to untreated cells were performed with one-way ANOVA, where $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

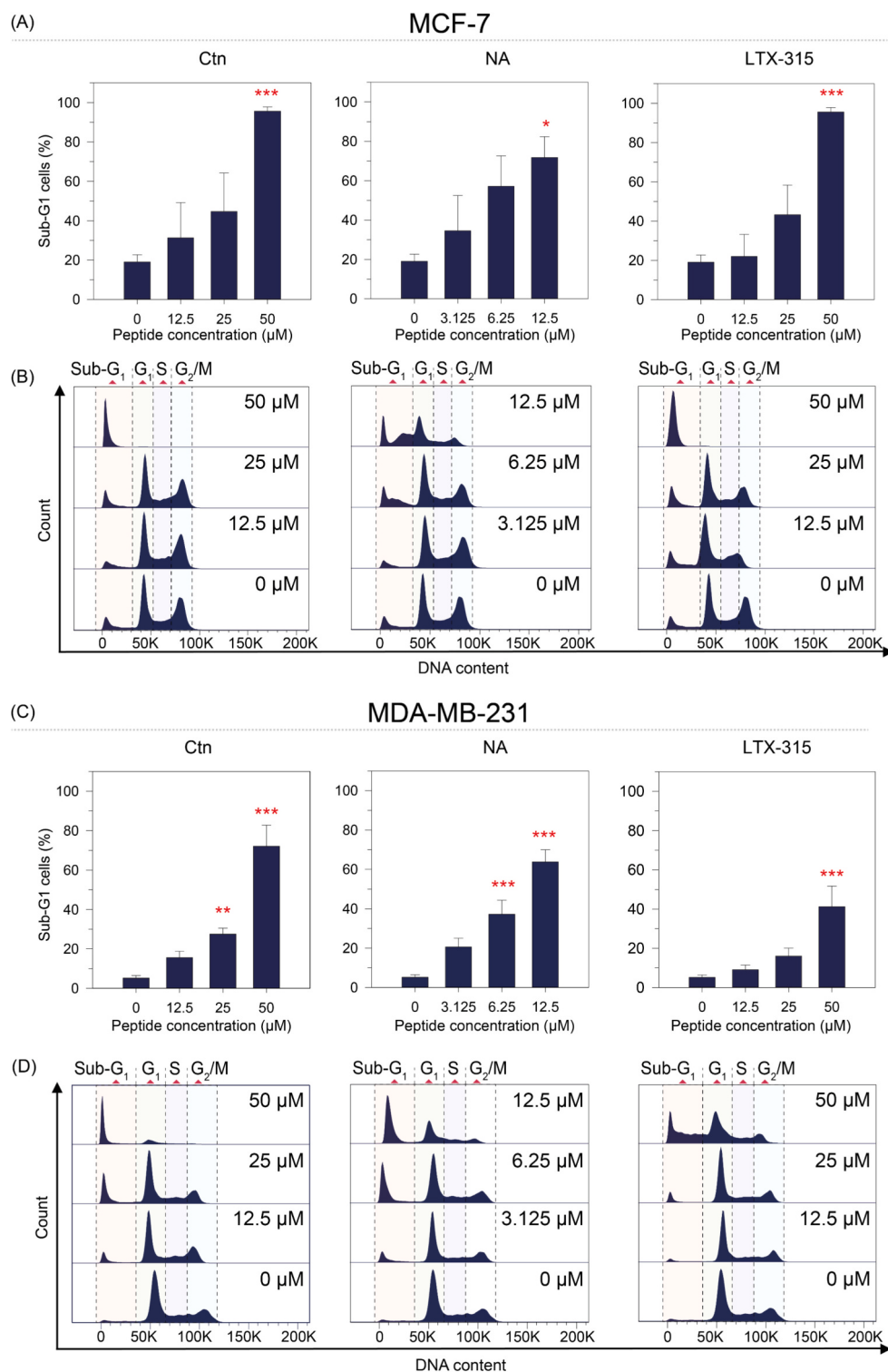


Fig. 3. Effect of Ctn, NA, and LTX-315 peptides on sub-G1 population of MCF-7 and MDA-MB-231 breast cancer cells. The cells were treated with various concentrations of the peptides for 6 h, stained with propidium iodide, and analyzed using flow cytometry to assess changes in cell cycle phase distribution or induction of sub-G1 population. The Sub-G1 cell population, indicative of DNA fragmented content, was quantified in (A) and (C). The Sub-G1 cell population is specifically highlighted in the bar graphs. The figures in (B) and (D) represent DNA content histograms of breast cancer cells following treatment with the respective peptides. All data are presented as the mean $\pm$ SEM ($n = 4$). One-way ANOVA compared the Sub-G1 population between untreated cells (0 μ M) and treated cells. Statistical significance is denoted by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

The incubation of the peptides with the lipid extract showed that in all cases, the three peptides interact with the lipids present in the cell membranes of HaCaT, MCF-7 and MDA-MB-231 by inducing changes in the thermotropic behavior of the lipid systems. A similar interaction

with membrane lipids from the three cell lines was observed with LTX-315, as shown in Fig. S6. The FT-IR results are associated with the viability results obtained by MTT. NA induced the most potent destabilization effect on the lipid systems. These results are also related to

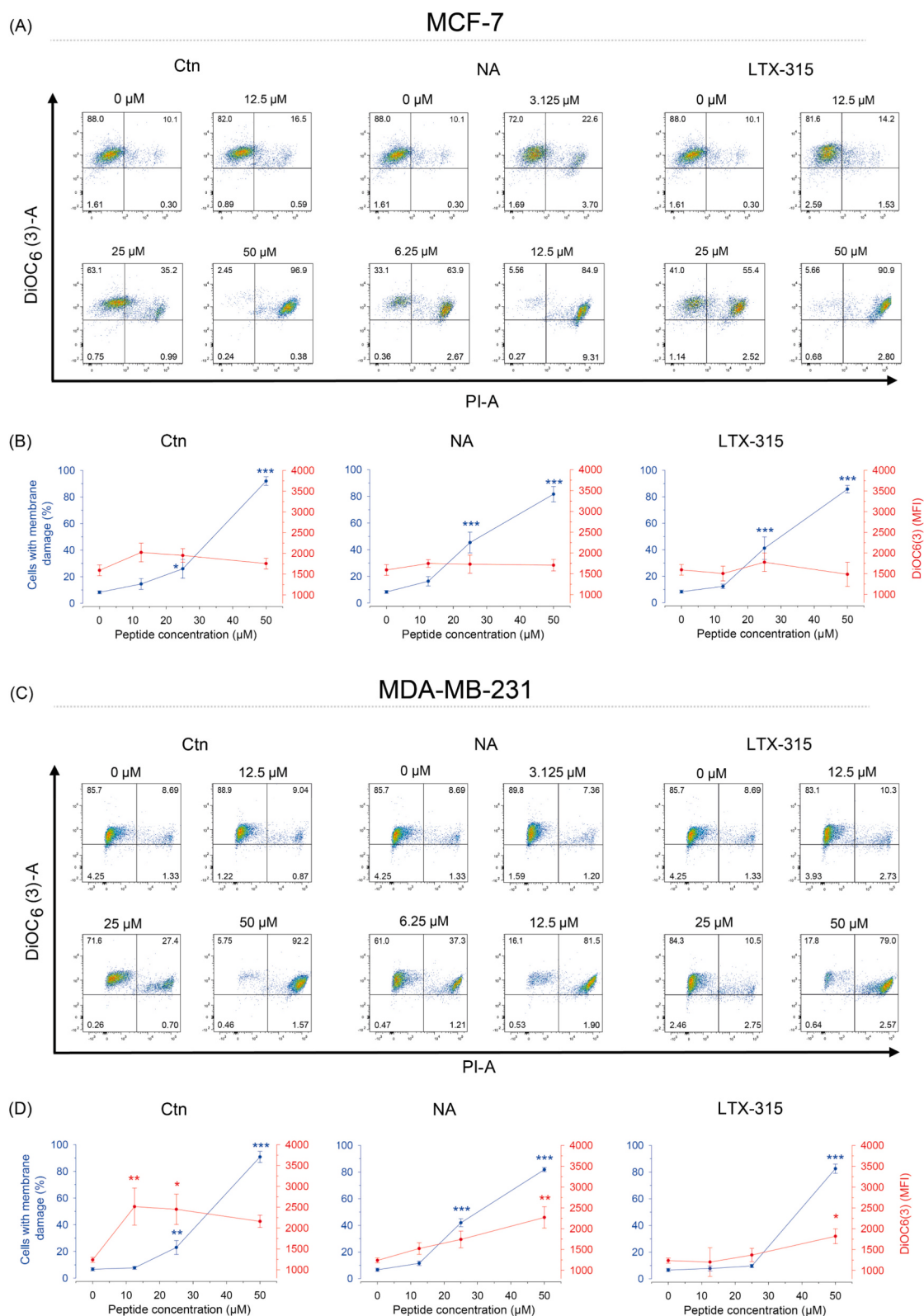


Fig. 4. Effect of Ctn, NA, and LTX-315 peptides on plasma membrane integrity and mitochondrial functionality. MCF-7 and MDA-MB-231 breast cancer cells were treated for 6 h with increasing concentrations of Ctn, NA, or LTX-315. (A) and (C) Representative dot plots show the distribution of cell populations based on DiOC₆(3)/PI staining in MCF-7 and MDA-MB-231 cells, respectively. The quadrants represent: DiOC₆(3)-high/PI-negative (viable cells), DiOC₆(3)-low/PI-negative (cells with mitochondrial depolarization and intact membranes), DiOC₆(3)-low/PI-positive (cells with mitochondrial depolarization and membrane compromise), and DiOC₆(3)-high/PI-positive (cells with preserved mitochondrial potential and membrane disruption). (B) and (D) Quantification of DiOC₆(3) mean fluorescence intensity (MFI, right axis -●-) and percentage of PI-positive cells (left axis -●-) in MCF-7 and MDA-MB-231 cells, respectively. Data represents the mean ± SEM of four independent experiments. Statistical significance compared to untreated cells (0 μM) was determined using one-way ANOVA (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). **Note:** (A) and (C) represent independent experiments for each cell line, with distinct control dot plots for MCF-7 and MDA-MB-231. Within each cell line, the same control (0 μM) dot plot is used across peptide treatments, as these are part of a larger experiment, and a representative image is shown for each condition.

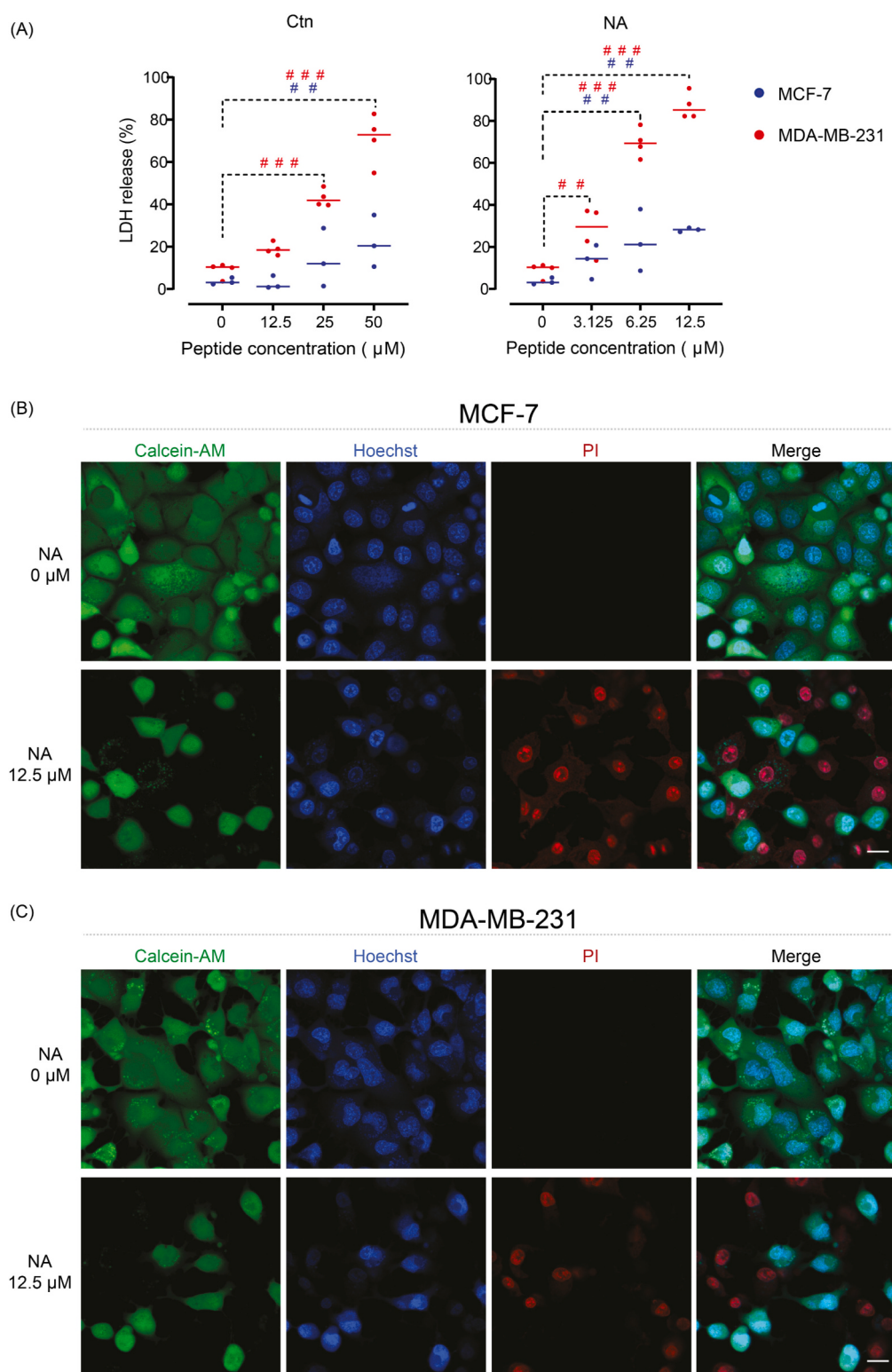


Fig. 5. Evaluation of the membrane-disrupting activity of Ctn and NA against breast cancer cells after 6 h of treatment. (A) LDH release into the culture medium was measured in MCF-7 (●) and MDA-MB-231 (●) cells treated with increasing concentrations of Ctn and NA (3.125–50 μ M). Values were normalized by the maximum LDH release control (10 % Triton X-100). Data are presented as mean $\pm$ SEM from at least three independent experiments performed in duplicate. Statistical significance was determined using one-way ANOVA compared to untreated control (0 μ M) where # p < 0.05, ## p < 0.01, ### p < 0.001. Representative confocal microscopy images of (B) MCF-7 and (C) MDA-MB-231 cells, untreated (0 μ M) or treated with NA (12.5 μ M). Blue fluorescence indicates Hoechst 33342-stained nuclei, green fluorescence represents Calcein-AM live cells, and red fluorescence shows PI uptake, marking cells with membrane damage. Images were acquired at 20 $\times$ magnification using the CD7 confocal microscope. Scale bar: 20 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

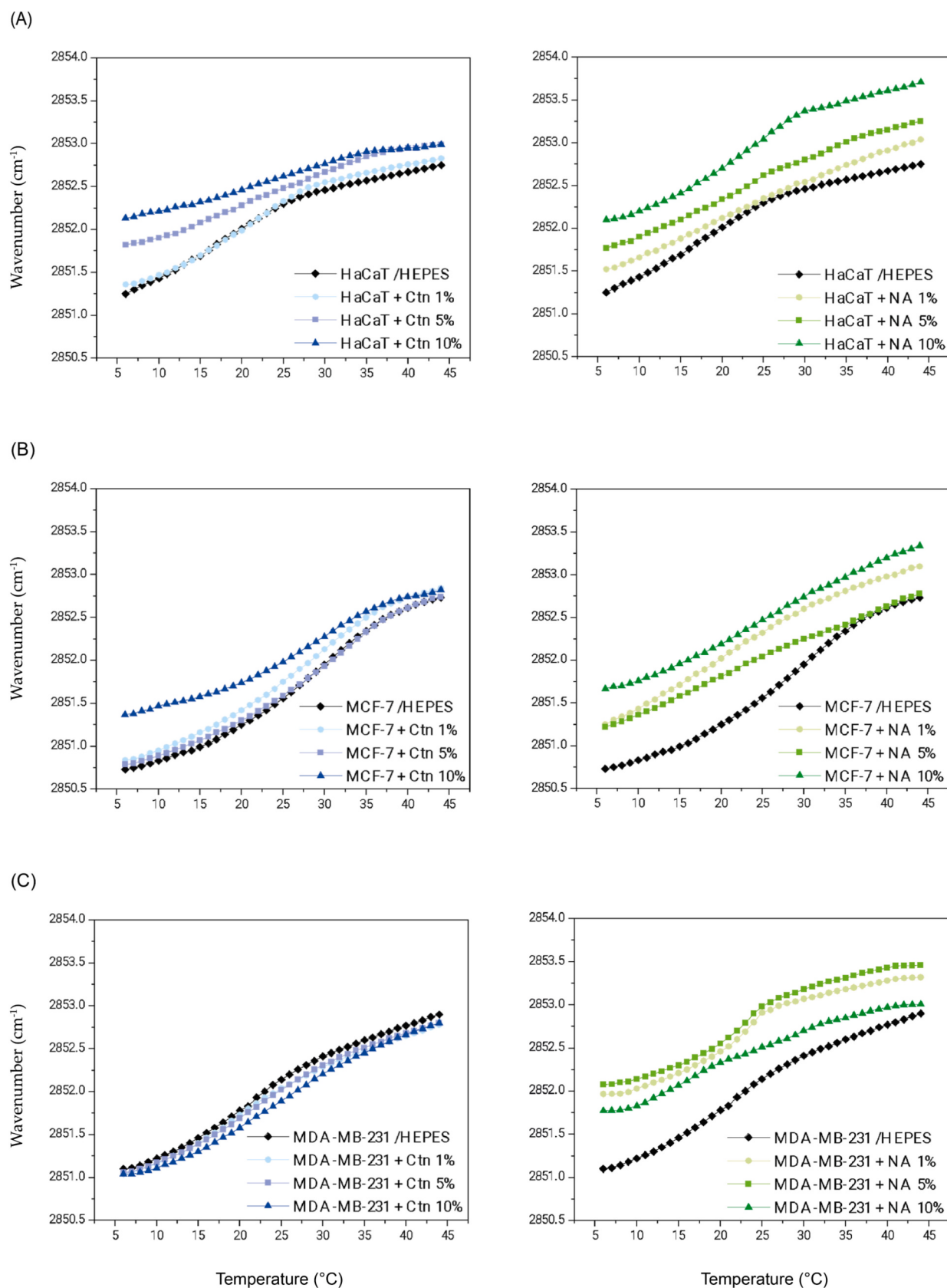


Fig. 6. Peak positions of symmetric stretching vibration band of the methylene groups as a function of temperature. Effect of increasing concentrations of Ctn (—) and NA (—) in the (A) HaCaT, (B) MCF-7, and (C) MDA-MB-231 lipid extracts. All experiments were performed as three independent measurements.

results obtained with the LDH assay where NA presented a higher membrane-disrupted activity than Ctn (Fig. 5).

4. Discussion

In the last 20 years, an increasing number of studies have been reported on membrane active peptides. These peptides exert their biological activity by interacting with the cell membrane, either to disrupt it and lead to cell lysis or to translocate through the cell. CPs are prominent as therapeutic agents owing to the generation of cancer resistant cells to current chemotherapeutics. The present study demonstrated the cytotoxic activity of synthetic peptides Ctn and NA in MCF-7 and MDA-MB-231 cell lines, representing two different breast cancer subtypes, luminal and TNBC, respectively [42]. Our results suggest that Ctn and NA induce non-apoptotic cell death, likely through a membrane-disrupting effect, similar to the control peptide LTX-315. These findings indicate that Ctn and NA exhibit similar membrane-lytic properties as LTX-315, with NA being the most active peptide.

The results revealed that all peptides caused a significant membrane-disrupting effect in MDA-MB-231 and MCF-7 cells. Both breast cancer cell lines exposed to the highest Ctn or NA peptide concentration exhibited necrotic morphological features, as was previously reported for LTX-315 peptide [38]. At the highest peptide doses evaluated, we also observed significant decreases in size and increases in granularity. Additionally, we detected severe damage to the cell membrane, characterized by increased DNA fragmentation, significant release of the intracellular enzyme LDH, and high proportions of PI-positive cells with compromised membranes within the population. These findings support the disruption of the membrane integrity induced by Ctn and NA and align with previous studies on antimicrobial peptides in human tumor cells, which have shown evidence of non-apoptotic cell death. This alternative cell death, distinct from classical apoptosis, suggests a 'necrosis-like' mechanism induced by these peptides, likely involving membrane disruption and cellular disintegration [37,38,43]. We found no evidence that Ctn or NA explicitly targets the mitochondria or induces apoptosis. CPs like LTX-315 have been identified as agents capable of entering cells to exert physiological effects and cause damage to mitochondria. However, previous studies by Zhou et al. have shown that LTX-315 internalization and accumulation close to mitochondria occur only at subtoxic concentrations. At high cytotoxic concentrations (100 µg/ml, ~ equivalent to 69 µM), LTX-315 triggers necrotic cell death [38].

We found that both peptides had a substantially higher cytotoxic impact, especially on MDA-MB-231 cells, than non-tumoral ones. However, it is important to note that non-tumorigenic HaCaT cells were also susceptible to the cytotoxic actions of these peptides. This non-selective effect was also reported for LTX-315 in human normal cell lines, including the endothelial cell line HUVEC and the fibroblast cell line MRC-5 [19,44]. To mitigate this off-target toxicity, a promising approach is an intratumoral administration, as demonstrated with the LTX-315 peptide. Intratumoral approaches can enhance drug dose delivery and bioavailability within the tumor microenvironment, by directly delivering the peptide to the tumor site, LTX-315 can exert its potent antitumor effects [45] while minimizing systemic exposure to healthy tissues, thereby reducing unintended cytotoxicity and enhancing local membranolytic activity [46,47]. It was reported that peptide recruitment precedes membrane permeabilization [48].

The composition of the cell membrane lipids can influence the activity of CPs. Membrane bilayers in different cell lines exhibit variations in composition, properties, and functions and may underlie the selective actions of CPs [49]. In a recent study, we described that the PS membrane composition of MDA-MB-231 cells is similar to those of HaCaT, but here, we still observed a two- to three-fold higher sensitivity in MDA-MB-231 [49], which represents the TNBC subtype. The observed heightened sensitivity of MDA-MB-231 cells to Ctn and NA, even surpassing LTX-315, suggests their potential as therapeutic agents against

the challenging TNBC subtype. Interestingly, MCF-7 cells have a much lower PS content than HaCaT cells but showed similar sensitivity towards these peptides as HaCaT cells, pointing to the importance of the PS content of tumor cells for peptide sensitivity [49]. In addition to PS, cell surface sialic acid content in cancer cells can favor the attraction of CPs to anionic membranes [50]. It has been reported that the MDA-MB-231 cell line has a higher level of α -2,3-sialic acid residues compared to the MCF-7 cell line [51]. This characteristic, in addition to the difference in PS levels, could contribute to the greater sensitivity of MDA-MB-231 cells to the peptides evaluated.

The mechanism by which CPs execute their function depends on several physicochemical properties, including the amino acid sequence, net charge, amphipathicity, hydrophobicity, peptide concentration, and membrane composition [16]. Ctn and NA share physicochemical properties; they are +15 charged peptides at physiological pH and have a 34-amino acid sequence. According to the FT-IR results, both Ctn and NA had an affinity for the lipids present in the total lipid extract of HaCaT, MCF-7, and MDA-MB-231, as evidenced by the modulation of the phase transition temperature in the lipid system. These findings suggest that the peptides directly interact with membrane lipids, inducing a destabilizing effect consistent with the membrane damage observed in the LDH release assay. Notably, NA exhibited the highest potential in disrupting the lipid packing of the membrane, causing significant changes in the phase transition temperature of the control (SLBs hydrated with 10 mM of HEPES). Further investigation is needed to determine whether this difference is the result of higher NA activity compared to Ctn.

Based on the results of this study, Ctn and NA mainly exert their antitumor activity on breast cancer cells through necrotic effects by disrupting the plasma membrane. This type of tumor cell death can influence anti-tumor immunity [52]. LTX-315 has been reported to induce immunogenic cell death through an unregulated necrotic pathway that triggers immune responses [53,54]. Further studies are needed to determine whether the necrotic activity of Ctn and NA exhibits hallmarks of immunogenic cell death.

Peptides that directly disrupt cell membranes without targeting specific receptors may offer certain advantages over chemotherapy. One key benefit is their rapid mechanism of action—by destabilizing the membrane, inducing cell death. This non-specific approach helps prevent the potential development of resistance in cancer cells. If the peptides are administered via intratumoral injection, these peptides could quickly eliminate tumor cells and halt tumor growth. Additionally, membrane-disrupting peptides can be explored in synergy with traditional chemotherapeutics. At low concentrations, they can be co-administered with chemotherapy drugs to enhance membrane permeability, facilitating drug uptake and enhancing therapeutic efficacy. Exploring the potential of combining these peptides with other drugs, such as chemotherapeutics or immune checkpoint inhibitors, could enhance treatment efficacy and mitigate the risk of tumor resistance development. However, a significant drawback of membrane-disrupting peptides is the need for precise intratumoral administration to minimize potential cytotoxic effects on healthy host tissues.

5. Conclusions

Our findings reveal that Ctn and NA-CATH-ATRA-1-ATRA-1 (NA) exhibit significant cytotoxic effects on MCF-7 and MDA-MB-231 cancer cells, with NA demonstrating superior potency compared to Ctn and surpassing the effectiveness of the clinical-phase control peptide LTX-315. NA has not been evaluated in cancer cells until now. Ctn and NA interact with membrane lipids in breast cancer cell lines, leading to membrane destabilization, as evidenced by plasma membrane disintegration, altered cell permeability, and LDH release.

The primary mode of action for Ctn and NA is direct membrane disruption rather than the induction of apoptosis. This unique ability to effectively disrupt cancer cell membranes presents a promising novel approach to breast cancer treatment. The membrane-targeting

mechanism of Ctn and NA could offer a significant advantage in overcoming the resistance often seen with traditional therapies, providing a complementary strategy to existing treatments.

The potential of Ctn and NA to enhance breast cancer treatment through membrane disruption warrants further investigation, particularly to elucidate the implications of their lytic effect. Understanding these effects could uncover new insights into their role in eliciting pro-immune responses similar to those reported for LTX-315. By leveraging this innovative mechanism, we may pave the way for more effective and resilient cancer therapies.

CRedit authorship contribution statement

Vanessa Gallego-Londoño: Formal analysis, Writing – original draft. **Gloria A. Santa-González:** Methodology, Formal analysis, Writing – review & editing. **Juan M. Giraldo-Lorza:** Investigation. **Marcela Manrique-Moreno:** Supervision, Project administration, Funding acquisition, Writing – review & editing. **Mauricio Rojas:** Writing – review & editing. **G. Bea A. Wisman:** Writing – review & editing. **Steven de Jong:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbmem.2025.184429>.

Data availability

No data was used for the research described in the article.

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