ANTICANCER RESEARCH *41*: 5415-5423 (2021)
doi:10.21873/anticanres.15353

Simplified Theta-defensin [Ser^{3,7,12,16}] RTD-2 Analog Is Involved in Proteasomal Degradation Pathway in Breast Cancer

JOANNA PIANKA[1#], NATALIA GRUBA[1#], KAMILA KITOWSKA[2], KAMIL MIECZKOWSKI[2], MAGDALENA WYSOCKA[1], RAFAŁ SĄDEJ[2] and ADAM LESNER[1]

[1]*Department of Environmental Technology, Faculty of Chemistry, University of Gdansk, Gdansk, Poland;*
[2]*Department of Molecular Enzymology and Oncology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Gdansk, Poland*

Abstract. *Background/Aim: Antimicrobial peptides are part of the innate immune response, regulate inflammation and initiate acquired immunity. This study focused on theta-defensins that have been shown to have anticancer properties. Materials and Methods: RTD-2 analogs were synthesized on a peptide synthesizer. Cell viability was estimated using the MTT test. Immunoprecipitation assay was conducted to determine the molecular partner of the [Ser3,7,12,16]-RTD-2 analog. Results: Here, we present the biologically active [Ser3,7,12,16]-RTD-2 analog that selectively targets various types of breast cancer cells. Immunoprecipitation protein-protein interaction studies showed eleven proteins common to MDA-MB-231 and T47D cell lines. Taking into account their cellular location, it can be concluded that the synthesized peptide interacts mainly with nuclear proteins, which correlates with the obtained microscopic image. Conclusion: Proteins that interact strongly with the [Ser3,7,12,16]-RTD-2 analog are closely related to the proteasomal protein degradation pathway. As the activity of the ubiquitin-proteasome system is markedly increased in patients with breast cancer, it is likely that selective modulation of this system may be a useful method for breast cancer treatment.*

Defensins are naturally occurring antimicrobial peptides, which are a part of the innate immune system, protecting the body from foreign microorganisms. They are produced by the interaction of antigen-presenting microbial cells with pattern-recognizing receptors, such as toll-like receptors, which are present on the membrane of many immune cells (*i.e.*, macrophages, neutrophils, and leukocytes) (1). They occur in most multicellular creatures - both in plants, fungi, insects, mollusks, arachnids, and mammals, including humans (2). Regardless of their origin, they show structural and functional similarities. Like other AMP peptides, most defensins interact with the components of the microbial cell membrane to have antibacterial, antiviral, and antifungal properties. These cationic, multifunctional peptides play an important regulatory function in the immune system, both innate and adaptive. Apart from the classic antimicrobial function, they play a role in immune modulation, fertility, development, and wound healing (3).

Theta-defensins are the smallest peptides among the defensins. They were discovered in 1999, when the macaque extract (*Macaca mulatta*) was purified and its components tested for bactericidal activity (4). In macaque neutrophils, six θ-defensin isoforms were expressed and showed antimicrobial activities against *E. coli, S. aureus,* and *C. albicans* (5). Interestingly, such compounds have been found in Old World monkeys, but not in higher primates including gorillas, chimpanzees, and humans (6, 7).

Rhesus theta defensins (RTDs) chain consists of only 18 amino acid residues and three disulfide bonds in a I–VI, II–V, III–IV arrangement (8, 9). The cyclic structure of θ-defensins is crucial for their activity due to the fact that it provides high stability, resistance to proteases, and antibacterial activity (8, 10).

Defensins have the potential to be used as biomarkers and therapeutic agents in the treatment of cancer. Studies have confirmed elevated levels of HNP-1, HNP-2, and HNP-3 in the serum of patients with colorectal cancer (11). Other cases of over-expression of human defensins include lung cancer patients with identified high levels of hBD-1 and hBD-2,

[#]These Authors contributed equally to this work.

Correspondence to: Natalia Gruba, Ph.D., University of Gdansk, Faculty of Chemistry, Laboratory of Biochemical Analysis and Nanodiagnostics, Wita Stwosza 63 Street, 80-308 Gdansk, Poland. Tel: +48 585235490, e-mail: natalia.gruba@ug.edu.pl

Key Words: Theta-defensins, breast cancer, immunoprecipitation, cell culture.

kidney cancer (high hBD-1), and oral squamous cell carcinoma (OSCC) (hBD-3 over-expression) (12). Human alpha defensins such as HD-5 have also been shown to act as biomarkers of inflammation in the human nasal mucosa and Crohn's disease (13).

Theta-defensins, mainly RTD-2, induce the proliferation of neoplastic cells, but importantly they are non-toxic to "normal" mammalian cells (14, 15). So far, it has been shown that simplified serine-rich RTD-2 peptide analogs are selective for breast cancer cells (14). The conducted experiments confirmed the inhibition of their growth in 3D culture, and the peptide concentrations used did not affect the growth of non-transformed cells (15).

Although *in vitro* studies have indicated that RTD peptides may be cytostatic against cancer cells, their *in vivo* use is still questionable. This is due to pharmacokinetic problems related to the delivery of potential molecules into the bloodstream, the cytotoxicity to "normal" cells at higher concentrations and the very high production costs of this type of drugs (16). However, this does not change the fact that further research on this interesting group of compounds may lead to their safe use in oncological therapy.

The aim of this study was to synthesize simplified RTD-2 analogs modified at positions 3, 7, 12, and 16 with a set of hydrophilic amino acid residues, and then to study their anticancer properties. Designing defensin-based peptides with high anti-tumor selectivity, efficacy and low cytotoxicity against untransformed epithelial cells would be an important step in the development of peptide drug design tools and a new type of therapy. For this purpose, we conducted *in vitro* studies based on a panel of cell lines derived from advanced stages of breast cancer (MDA-MB-231, SKBR3, T47D cell lines).

Materials and Methods

Peptide synthesis. [Arg^{3,7,12,16}]-RTD-2, [Asn^{3,7,12,16}]-RTD-2, [Ser(Me)^{3,7,12,16}]-RTD-2, [Ala^{3,7,12,16}]-RTD-2, [Lys^{3,7,12,16}]-RTD-2, [Thr^{3,7,12,16}]-RTD-2, [Ser^{3,7,12,16}]-RTD-2, [Ser^{3,7,12,16}]-Ala(2-BAD)RTD-2, and [Ser^{3,7,12,16}]-Lys(HOC)RTD-2 were synthesized on solid phase using a Liberty Blue™ peptide synthesizer (CEM GmbH, Kamp-Lintfort, Germany) in accordance with a standard protocol based on Fmoc/tBu chemistry. The solid support was H-L-Arg(Pbf)-2-chlorotrityl resin (Iris Biotech, Marktredwitz, Germany) with a deposition of 0.63 mmol/g. Briefly, peptides were synthesised by repeating deprotection and acylation steps. Fmoc protecting group was cleaved by the use of 30% piperidine in dimethylformamide (DMF), while Oxyma/diisopropylcarbodiimide (DIC) method was used for coupling reactions. Upon completion of the synthesis, the *N*-terminal Fmoc group was removed and peptides were cleaved from the resin with the simultaneous removal of the side protecting groups by treating with a mixture of TFA, phenol, triisopropulsiane and H_2O (88:5:2:5, v/v) (17). Peptides were oxidized and cyclized according to a procedure previously described (14).

Obtained compounds were analysed on a high performance liquid chromatography Jasco LC System (Jasco, Tokyo, Japan) on RP C8 column (Supelco Wide Pore C8 column 8×250 mm, Phenomenex, Aschaffenburg, Germany) with linear gradient of 10%-90% buffer B (80% acetonitrile, 19.9% water, 0.1% TFA) in buffer A (0.1% TFA in water) for 40 min at a flow rate of 1 ml/min. Synthesized peptides were purified on a semipreparative Luna C18 column (Supelco Wide Pore C18, 5 μm, 250 mm ×10 mm, Phenomenex). Analysis of mass ions was performed on autoflex maX MALDI TOF mass spectrometer (Bruker, Bremen, Germany).

Table I. *Components of the buffer used for immunoprecipitation.*

Compound	Concentration
PMSF	2 mM
EGTA	5 mM
Aprotynin	10 μg/ml
Leupeptin	10 μg/ml
EDTA	1 mM
$Na_4P_2O_7$	2 mM
NaF	5 mM
Na_3VO_4	5 mM

Cell culture. HB2 mammary epithelial cells were purchased from ECACC (Sigma-Aldrich, Darmstadt, Germany), MDA-MB-231 and SKBR3 were purchased from ATCC (Manassas, VA, USA), T47D cells were obtained from DSMZ (Braunschweig, Germany). Cells were maintained in DMEM (Life Technologies, Gibco, Waltham, MA, USA) with 10% FBS (fetal bovine serum) (Life Technologies, Gibco) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). The medium for HB2 cells was supplemented with insulin (5 μg/ml) and hydrocortisone (5 μg/ml) (Life Technologies, Gibco). Cells were grown at 37˚C in a humidified atmosphere of 5% CO_2, passaged for a maximum of 3-4 months post resuscitation, and regularly tested for mycoplasma contamination. Cells were passaged when they reached 80% confluency.

Cytotoxicity assay. Cell viability was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT) colorimetric assay (18, 19). The cells were seeded at a concentration of 7-9×10^3 cells per well in 96-well plates in triplicates. After 24 h, the medium was changed to a medium containing the appropriate concentration of tested compounds (100 μg/ml, 25 μg/ml and 5 μg/ml) and incubated at 37˚C, 5% CO_2 for 48 h. MTT stock solution was added to each well so that the final concentration of MTT in the medium was 0.5 mg/ml and the cells were incubated for 2 h. Cell viability was estimated by measuring the absorbance at 590 nm using a microplate reader. Each measurement was repeated at least three times, and the percentage of viable cells was calculated by comparing the absorbance values of the test samples with that of the untreated cells (control).

Culturing cells in three-dimensional Matrigel®. Cell growth in three-dimensional Matrigel® (BD Bioscience Matrigel Matrix Growth Factor Reduced) was carried out as previously described (20, 21). Cells were cultured in regular medium or medium containing the tested compounds (final concentration 10 μg/ml). Media were changed every third day. After 10-14 days of culture (depending on the cell line) cell growth was evaluated by measuring the size of the colonies (at least 100) using ZEISS PrimoVert microscope (ZEISS, Esslingen, Germany) and ImageJ software (LOCI, WI, USA).

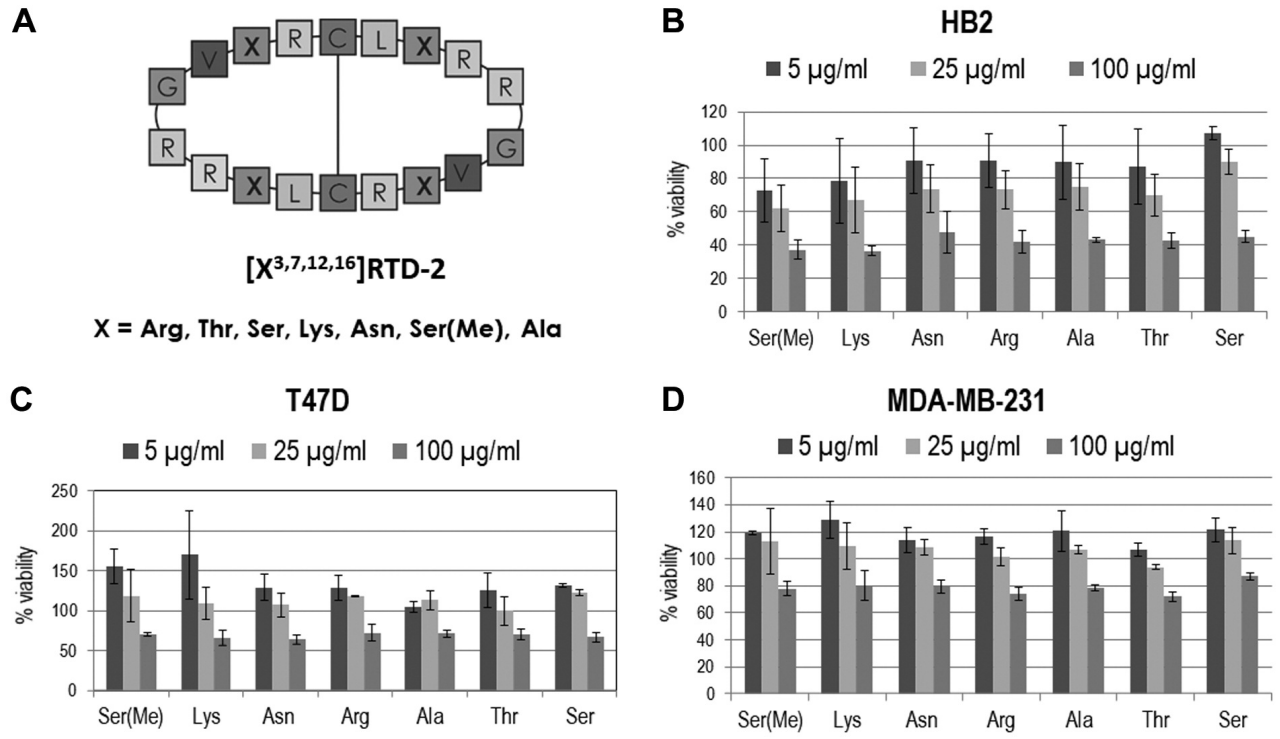


Figure 1. *Cytotoxicity assays. A) Structure of RTD-2 analogs; B, C, D) 2D test results (MTT) against the HB2, T47D, and MDA-MB-231 breast cancer lines, where 100% is the control (untreated cells). Each of the experiments was repeated three times.*

Immunoprecipitation. Immunoprecipitation assay was performed for [Ser^{3,7,12,16}]-Lys(Bt)RTD-2 analog. MDA-MB-231 and T47D cells were seeded in 10 cm diameter dishes. The next day, the medium was replaced with the culture medium supplemented by the tested compound. After 4 h, the cells were washed 3 times with sterile PBS and lysed with the use of immunoprecipitation buffer containing protease inhibitors (Table I).

Cells were then removed from the dishes and transferred to Eppendorf tubes. The cell homogenate was centrifuged at 14,000 × *g* for 2 min at 4˚C. Then, the supernatant was removed and a mixture of Triton X-100 detergent with inhibitors was added. The cell lysates prepared in this way were left on the stirrer at 4˚C overnight. Later, the mixture of cells was centrifuged again, maintaining the same conditions, the supernatant was collected, and 60 µl of avidin beads was added. Samples were incubated for 24 h at 4˚C. The next day, cells were washed 2 times with PBS, centrifuged, and the supernatant was removed. One ml of the mixture of Triton X-100 with protease inhibitors was added to the residue in the test tubes, mixed, and centrifuged again. One hundred µl of Laemmli denaturing loading buffer [62.5 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 100 mM DTT, 0.2 mM bromophenol blue] was added and left in a cooler on a stirrer for 20 min. After this, the tubes were placed in a centrifuge for 3 min, and the obtained supernatant was transferred to clean tubes. After elution of the protein with the bed, the mixture was precipitated with a chloroform-methanol system (22) and sent in the form of a pellet for analysis to the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw.

Statistical analyses. All data were analyzed with the use of Graph Pd Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Experiments were carried out in duplicate or triplicate and are presented as the mean±standard error (SEM). The Mann-Whitney test was used for the statistical analysis.

Results

In the previous work (14) it was demonstrated that among synthesized simplified θ-defensins, RTD-2 serine derivative, showed promising anticancer activity. Here, we decided to go a step further and investigate cellular localization of RTD-2 serine derivatives and identify a molecular partner that interacts with them. First, the impact of seven simplified RTD-2 peptide analogs on cell proliferation based on MTT assay was assessed. The cytotoxicity tests were conducted on three breast cancer cell lines: T47D, MDA-MB-231, and HB2. The latter are untransformed epithelial cells that were treated as control. RTD-2 analogues used in the cytotoxicity tests showed satisfactory efficacy against the breast cancer cells (Figure 1). The most sensitive were HB2 cells, for which a decrease in viability by at least 10% was observed at the lowest concentration of the analogues used. However, only the highest concentration of the peptides affected the viability of

T47D and MDA-MB-231. As the obtained results were unsatisfactory, we additionally performed three-dimensional cell cultures in Matrigel®, because they better reflect the tumor environment and more reliably illustrate the response to drugs. The results of cell responses to individual θ-defensins are presented in Figure 2. The derivative [$Arg^{3,7,12,16}$] RTD-2 resulted in approximately 40%, 50% and 30% reduction of SKBR3, HB2, and MDA-MB-231 cell proliferation, respectively (at the lowest concentration used in the experiment). The peptide [$Thr^{3,7,12,16}$] RTD-2 induced an approximately 60%, 50% and 45% reduction of SKBR3, HB2, and MDA-MB-231 cell proliferation, respectively. In contrast, incubation with [$Ser^{3,7,12,16}$] RTD-2 resulted in approximately 70% reduction in colony formation by MDA-MB-231 cells. For the SKBR3 cells, the colony size was reduced by approximately 40%. In the case of HB2 untransformed cells (control), we observed an approximately 50% inhibition of cell growth was observed. Based on these observations, it can be concluded that the analogs show significant anti-tumor potential at concentrations as low as 5 μg/ml.

The conducted studies confirmed that the simplified RTD-2 peptide, rich in serine residues significantly inhibited the proliferation of MDA-MB-231 cells at a concentration of 5 μg/ml. We also performed an experiment in which we used additional concentrations of the peptide [$Ser^{3,7,12,16}$] RTD-2 to determine the lowest possible effective dose against the MDA-MB-231 line (Figure 3). A comparable effect of about 70% decrease in cell viability was obtained at a five times lower concentration.

In the next step, we designed and synthesized new fluorescently labeled [$Ser^{3,7,12,16}$] RTD-2 analogs. To determine the potential antitumor activity of [$Ser^{3,7,12,16}$]-Ala(2-BAD) RTD-2 and [$Ser^{3,7,12,16}$]-Lys (HOC) RTD-2 analogs, we analyzed the growth of MDA-MB-231 cells in 3D cultures (Figure 4). The experiments were carried out using two RTD-2 analogs at concentrations of 5 μg/ml and 25 μg/ml. The obtained results show that both peptides caused significant toxicity to triple negative breast cancer cells. For the MDA-MB-231 cell line, we observed an approximately 70% inhibition of cell growth at the lowest concentration used. The same results were obtained when incubating cells with the [$Ser^{3,7,12,16}$] RTD-2 analogue. Furthermore, we investigated the subcellular localization of the peptides. For this purpose, MDA-MB-231 and T47D cells were incubated with fluorescently labeled RTD-2 for 1, 4, 8 and 24 h. Representative images are shown in Figure 5. Significant accumulation of [$Ser^{3,7,12,16}$]-Ala(2-BAD) RTD-2 was observed in both breast cancer cell lines after an hour, and the peptide was mainly found in the perinuclear compartment. In the case of [$Ser^{3,7,12,16}$]-Lys(HOC) RTD-2 peptide, the peptide was located mainly in the cell membrane.

Lastly, we performed immunoprecipitation of the [$Ser^{3,7,12,16}$]-Lys(Bt) RTD-2 analog to determine a potential molecular partner. The study used quantitative label-free (LFQ) assays to identify proteins and associated molecular partners/pathways that could be altered by the action of the RTD-2 analog on breast cancer cells. The mass spectrometry analysis revealed over 1,200 proteins. From the results obtained with the MDA-MB-231 and the T47D cell lines, 160 and 373 proteins, respectively, were found to be over-expressed. The data obtained also included nine proteins showing specificity for the RTD-2 analogue, common to both cell lines. It is therefore likely that [$Ser^{3,7,12,16}$] RTD-2 has a dual effect of increasing ligand levels and inhibiting the release of intra- and extra-cellular proteins, as proteins with reduced expression compared to the control have also been detected.

Discussion

A novel therapeutic strategy against neoplastic cells is the use of peptides. Physicochemical properties, amino acid composition and the addition of chemical groups in the sequence of compounds affect their conformation, lattice charge and secondary structure orientation. That, in turn affects the specific interaction with cells, as well as the ability of active compounds to penetrate the cell membrane. The discovery of antimicrobial peptides having potential to target cancer cells, triggered the research focused on the use of these compounds in anti-cancer therapy. AMPs exhibit a number of features that make them an extremely attractive tool in the search for treatment strategies. These include, a broad spectrum of activity, good solubility in water, and often the lack of drug resistance (23, 24). The greatest disadvantage of the pharmacological properties of peptides is their short half-life, which is related to the susceptibility to digestion by proteases. Therefore, θ-defensins, whose cyclic structure contributes to the high stability of the compounds, makes them ideal candidates for research into potential drug development. So far, such compounds have been tested mainly for their antibacterial action, however, there are some reports (25, 26) that suggest that they are also toxic to neoplastic cells.

The current work focused on the anti-cancer potential evaluation of simplified RTD-2 analogs against breast cancer cell lines. Studies have shown that the bioactivity of θ-defensins is independent of the number of disulfide bridges (27). Additionally, it has been shown that central binding is sufficient for the proteolytic stability of peptides, which is an important factor from a pharmacokinetic point of view (16). Therefore, the first stage of this work involved the synthesis of simplified RTD-2 analogs modified at positions 3, 7, 12, and 16 with a set of hydrophilic amino acid residues such as serine, threonine, lysine, arginine, aspartic acid, asparagine, and alanine. Previous experiments showed that the change of Cys to Ser influences the general properties of such a peptide (*e.g.*, hydrophobicity) and the tendency to form hydrogen bonds

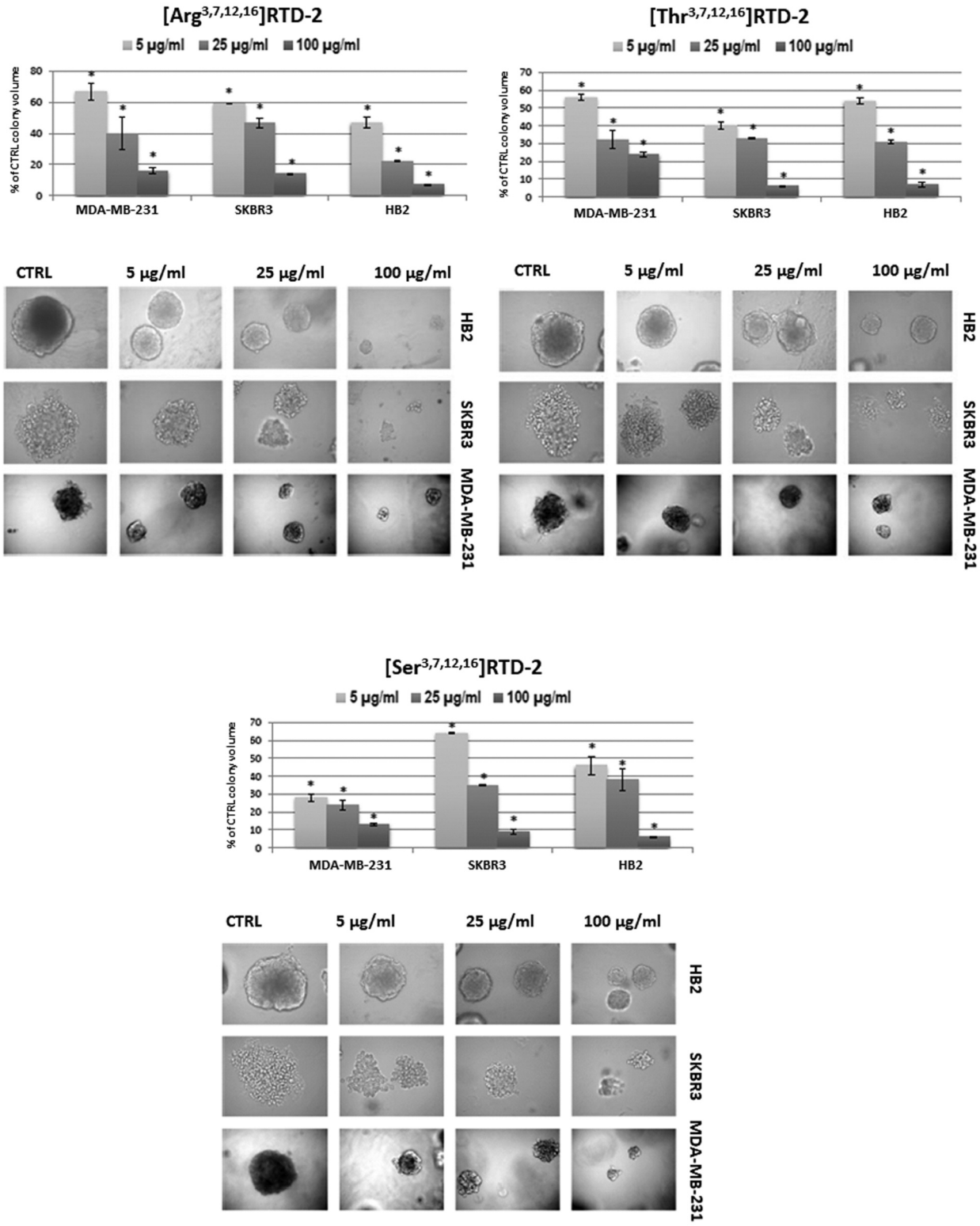


Figure 2. *Three-dimensional growth of MDA-MB-231, SKBR3, and HB2 cell lines in Matrigel® in medium containing: A) [Arg^{3,7,12,16}] RTD-2, B) [Thr^{3,7,12,16}] RTD-2 and C) [Ser^{3,7,12,16}] RTD-2 in the concentrations of 5 µg/ml, 25 µg/ml, and 100 µg /ml. The culture medium was changed every three days. At least 50 colonies for each condition were measured after 10 days of culture using a ZEISS PrimoVert microscope and ImageJ software. The effect of θ-defensin analogues was defined as the difference in colony size compared to the control. Results are expressed as the mean of three independent experiments. *p≤0.05.*

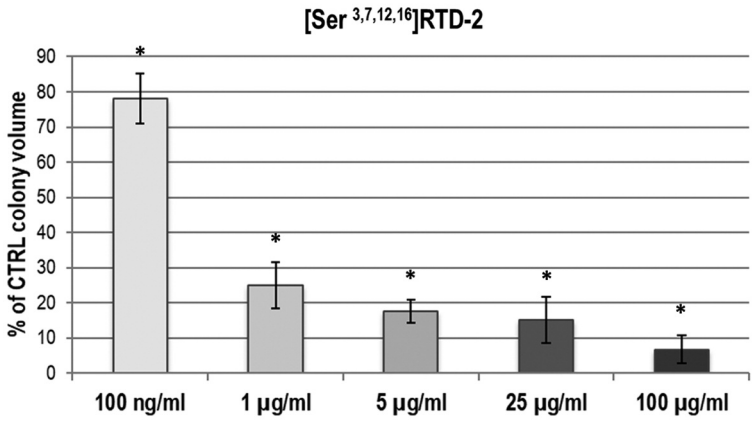
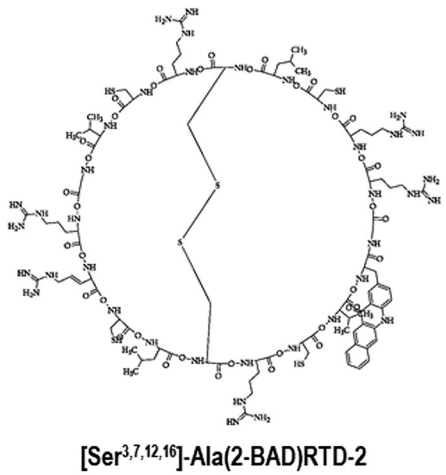
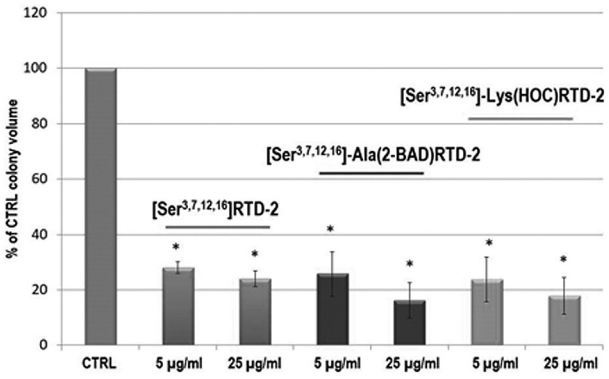
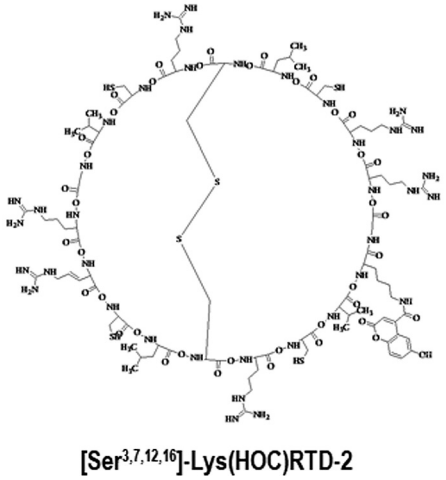


Figure 3. *Results of 3D assays of the MDA-MB-231 cell line in Matrigel® in medium containing various concentrations of [Ser^{3,7,12,16}] RTD-2 peptide. *p≤0.05.*







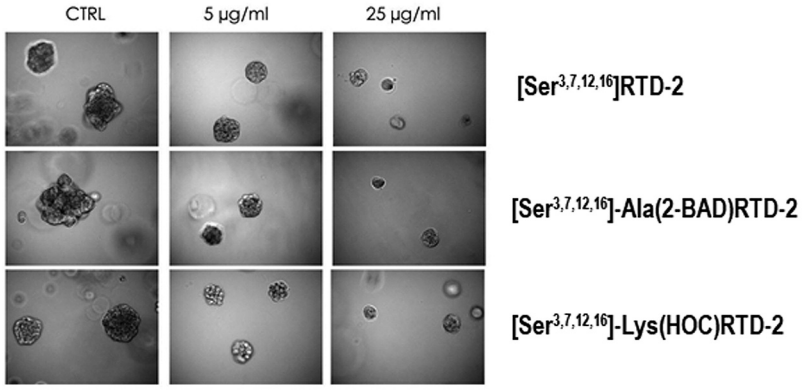


Figure 4. *Results of the three-dimensional growth of the MDA-MB-231 cell line in Matrigel® in medium supplemented with the following peptides: [Ser^{3,7,12,16}] RTD-2, [Ser^{3,7,12,16}]-Ala(2-BAD) RTD-2 and [Ser^{3,7,12,16}]-Lys(HOC) RTD-2 at the concentrations of 5 μg/ml and 25 μg/ml. The culture medium was changed every three days. To assess cell proliferation, colonies were measured after 10 days of culture (at least 50 colonies for each condition) using a ZEISS PrimoVert microscope and ImageJ software. The effect of theta-defensin analogs was defined as the difference in colony size compared to the control. Results are expressed as the mean of three independent experiments. *p≤0.05.*

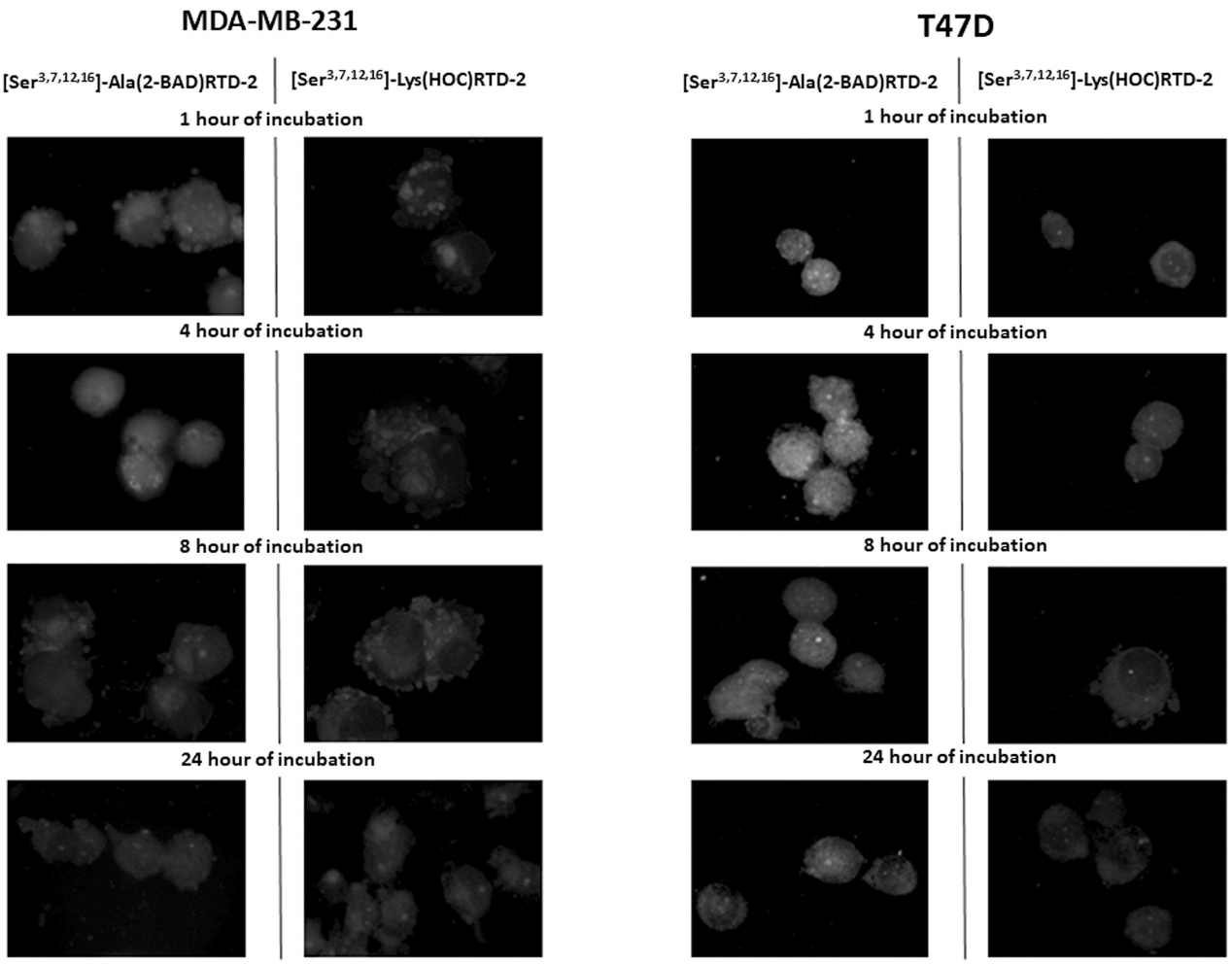


Figure 5. *Effect of RTD-2 analogs on cell localization in MDA-MB-231 and T47D cell lines. Cell growth was carried out in medium supplemented with [Ser^{3,7,12,16}]-Ala(2-BAD) RTD-2 and [Ser^{3,7,12,16}]-Lys(HOC) RTD-2 at a concentration of 5 μg/ml. For immunofluorescence experiments, cells were plated on coverslips and analyzed after 1, 4, 8, and 24 hours of incubation with the peptides, respectively. The images were captured using a Zeiss Axiovert 200 microscope coupled with an AxioCam ERc 5s camera (Zeiss). Scale: 100 μm.*

(14). Thus, based on the research described above, we decided to investigate whether this effect is limited only to a serine residue or another amino acid with similar properties. Seven simplified θ-defensin analogs, were subjected to biological analysis in order to evaluate their anti-cancer properties. Among them, [Ser^{3,7,12,16}] RTD-2 analog was found to be the most active. Fluorescently labeled [Ser^{3,7,12,16}]-Ala(2-BAD) RTD-2 and [Ser^{3,7,12,16}]-Lys (HOC) RTD-2 peptides show the same inhibitory effect on the growth of breast cancer cell lines (T47D, MDA-MB-231) as the unmodified simplified peptide rich in serine residues. By using these compounds, it was possible to assess the cellular localization of the tested peptides. Both designed peptides penetrated the cell interior at a concentration of 5 μg/ml. The [Ser^{3,7,12,16}]-Ala(2-BAD) RTD-2 analog was visible mainly in the vicinity of the cell nucleus, while [Ser^{3,7,12,16}]-Lys (HOC) RTD-2 was observed in most of the cell's internal structures after one hour of incubation. Analyzing the immunoprecipitation results obtained for the MDA-MB-231 and T47D lines (data not shown), we noticed that four out of all eleven proteins located in the cell nucleus are associated with the 26S proteasome. These include PSMD1, PSMD11, UBQLN1, and COPS4. The proteasome is a multi-protein complex involved in the ATP-dependent degradation of ubiquitinated proteins. It plays a key role in maintaining homeostasis by removing misfolded or damaged proteins that may interfere with cellular function. It is involved in the progression of the cell cycle, apoptosis or the repair of DNA damage (28). It consists of the 19S regulatory complex that directs ubiquitinated degradation substrates to the 20S catalytic core. PSMD1 is the largest subunit of the 19S

regulatory molecule and a key structural component that acts as a docking site for other subunits. PSMD11 is the lid of the 19S subunit. Many tumor suppressor and tumorigenic proteins are regulated by protein degradation mediated by ubiquitin and the proteosome (29). Therefore, PSMD1 plays an important role in regulating carcinogenesis and cancer progression. Recent studies showed that this protein is upregulated in anaplastic tumor of the thyroid gland and breast cancer, where it influences the regulation of proliferation and cell cycle progression by mediating the degradation of p53 protein in cells. Moreover, it may modulate the cell cycle profile in hormone-sensitive and tamoxifen-resistant breast cancer cells (30, 31). Similarly, ubiquilin-1 (UBQLN1), which is a ubiquitin-like compound, plays an important role in protein degradation. Disruption of its function affects the pathophysiology of neurodegenerative disorders (Alzheimer's disease, Huntington's disease) and cancer. Research has shown that this protein also promotes the development and progression of breast cancer, including the migration and invasion of neoplastic cells, and therefore, over-expression of UBQLN1 is a poor prognostic factor (32). *COPS4*, on the other hand, is a gene that codes for one of the eight subunits that make up COP9 signals - a protein complex that acts as an important regulator of many signaling pathways. Its structure and function are similar to those of 19S regulatory molecule of the 26S proteasome, as it interacts with ubiquitin ligases. It also plays an important role in protein degradation and regulatory processes such as cell cycle control, transcription activation or oncogenesis (33).

It has been shown that cisplatin, a widely used chemotherapeutic drug, which exerts a cytotoxic effect by affecting both nuclear and cytosolic pathways, inhibits the activity of the proteasome (34). The same situation exists in the case of doxorubicin, resulting in the accumulation of poly-ubiquitinated proteins and autophagosomes (35). Additionally, the compound induces DNA damage and leads to significant proteomic changes in neoplastic cells, making the ubiquitin-proteasome system a potential target for increasing the effectiveness of doxorubicin therapy. Although it is considered one of the most effective agents in the treatment of cancer, its use is severely limited due to severe side effects (36).

In summary, the proteins described above are important regulators of biological processes, and their expression profile may favor the assessment of therapeutic effects and disease prognosis. It is also worth noting that the simplified RTD-2 analog ([$Ser^{3,7,12,16}$] RTD-2) synthesized in this study enhances the effect of cisplatin and doxorubicin in breast cancer cell lines (14), which translates into a reduction in the effective dose of the drug, and consequently, reducing the undesirable side effects of therapy. Importantly, RTD-2 analog ([$Ser^{3,7,12,16}$] RTD-2) showed the strongest growth inhibitory effect against the highly aggressive MDA-MB-231 cell line, which is a model of triple negative breast cancer for which there are currently no effective targeted therapies. It is therefore possible that such a combination is a promising and effective alternative to chemotherapy-based cancer treatment.

Conflicts of Interest

There are no potential conflicts of interest related to this study.

Authors' Contributions

Conceptualization, A.L., R.S., J.P, N.G.; methodology, A.L., R.S., J.P, N.G; investigation, J.P., N.G., K.K., K.M., M.W.; writing-original draft preparation, J.P, N.G.; writing-review and editing, J.P., N.G., K.K., K.M., R.S., A.L.

References

1 Hazlett L and Wu M: Defensins in innate immunity. Cell Tissue Res *343(1)*: 175-188, 2011. PMID: 20730446. DOI: 10.1007/s00441-010-1022-4

2 Pearce G, Yamaguchi Y, Munske G and Ryan CA: Structure-activity studies of AtPep1, a plant peptide signal involved in the innate immune response. Peptides *29(12)*: 2083-2089, 2008. PMID: 18824048. DOI: 10.1016/j.peptides.2008.08.019

3 Esfandiyari R, Halabian R, Behzadi E, Sedighian H, Jafari R and Imani Fooladi AA: Performance evaluation of antimicrobial peptide ll-37 and hepcidin and β-defensin-2 secreted by mesenchymal stem cells. Heliyon *5(10)*: e02652, 2019. PMID: 31687504. DOI: 10.1016/j.heliyon.2019.e02652

4 Tran D, Tran PA, Tang YQ, Yuan J, Cole T and Selsted ME: Homodimeric theta-defensins from rhesus macaque leukocytes: isolation, synthesis, antimicrobial activities, and bacterial binding properties of the cyclic peptides. J Biol Chem *277(5)*: 3079-3084, 2002. PMID: 11675394. DOI: 10.1074/jbc.M109117200

5 Tongaonkar P, Tran P, Roberts K, Schaal J, Osapay G, Tran D, Ouellette AJ and Selsted ME: Rhesus macaque θ-defensin isoforms: expression, antimicrobial activities, and demonstration of a prominent role in neutrophil granule microbicidal activities. J Leukoc Biol *89(2)*: 283-290, 2011. PMID: 21084627. DOI: 10.1189/jlb.0910535

6 Selsted ME: Theta-defensins: cyclic antimicrobial peptides produced by binary ligation of truncated alpha-defensins. Curr Protein Pept Sci *5(5)*: 365-371, 2004. PMID: 15544531. DOI: 10.2174/1389203043379459

7 Cheng DQ, Li Y and Huang JF: Molecular evolution of the primate α-/θ-defensin multigene family. PLoS One *9(5)*: e97425, 2014. PMID: 24819937. DOI: 10.1371/journal.pone.0097425

8 Falanga A, Nigro E, De Biasi MG, Daniele A, Morelli G, Galdiero S and Scudiero O: Cyclic peptides as novel therapeutic microbicides: Engineering of human defensin mimetics. Molecules *22(7)*: 1217, 2017. PMID: 28726740. DOI: 10.3390/molecules22071217

9 Lehrer RI, Cole AM and Selsted ME: θ-Defensins: cyclic peptides with endless potential. J Biol Chem *287(32)*: 27014-27019, 2012. PMID: 22700960. DOI: 10.1074/jbc.R112.346098

10 Conibear AC and Craik DJ: The chemistry and biology of theta defensins. Angew Chem Int Ed Engl *53(40)*: 10612-10623, 2014. PMID: 25079086. DOI: 10.1002/anie.201402167

11 Kemik O, Kemik AS, Sumer A, Begenik H, Purisa S and Tuzun S: Human neutrophil peptides 1, 2 and 3 (HNP 1-3): elevated serum levels in colorectal cancer and novel marker of lymphatic and hepatic metastasis. Hum Exp Toxicol *32(2)*: 167-171, 2013. PMID: 21669914. DOI: 10.1177/0960327111412802
12 Al-Rayahi IA and Sanyi RH: The overlapping roles of antimicrobial peptides and complement in recruitment and activation of tumor-associated inflammatory cells. Front Immunol *6*: 2, 2015. PMID: 25657649. DOI: 10.3389/fimmu.2015.00002
13 Howell K and de Leeuw E: Cell adhesion properties of human defensins. Biochem Biophys Res Commun *502(2)*: 238-242, 2018. PMID: 29800568. DOI: 10.1016/j.bbrc.2018.05.150
14 Strzelecka P, Czaplinska D, Sadej R, Wardowska A, Pikula M and Lesner A: Simplified, serine-rich theta-defensin analogues as antitumour peptides. Chem Biol Drug Des *90(1)*: 52-63, 2017. PMID: 28004513. DOI: 10.1111/cbdd.12927
15 Makovitzki A, Fink A and Shai Y: Suppression of human solid tumor growth in mice by intratumor and systemic inoculation of histidine-rich and pH-dependent host defense-like lytic peptides. Cancer Res *69(8)*: 3458-3463, 2009. PMID: 19351852. DOI: 10.1158/0008-5472.CAN-08-3021
16 Kang SJ, Park SJ, Mishig-Ochir T and Lee BJ: Antimicrobial peptides: therapeutic potentials. Expert Rev Anti Infect Ther *12(12)*: 1477-1486, 2014. PMID: 25371141. DOI: 10.1586/14787210.2014.976613
17 Palomo JM: Solid-phase peptide synthesis: An overview focused on the preparation of bio-logically relevant peptides. ChemInform *45(47)*: 32658-32672, 2014. DOI: 10.1002/chin.201447272
18 van Meerloo J, Kaspers GJ and Cloos J: Cell sensitivity assays: the MTT assay. Methods Mol Biol *731*: 237-245, 2011. PMID: 21516412. DOI: 10.1007/978-1-61779-080-5_20
19 Stockert JC, Horobin RW, Colombo LL and Blázquez-Castro A: Tetrazolium salts and formazan products in Cell Biology: Viability assessment, fluorescence imaging, and labeling perspectives. Acta Histochem *120(3)*: 159-167, 2018. PMID: 29496266. DOI: 10.1016/j.acthis.2018.02.005
20 Mieszkowska M, Piasecka D, Potemski P, Debska-Szmich S, Rychlowski M, Kordek R, Sadej R and Romanska HM: Tetraspanin CD151 impairs heterodimerization of ErbB2/ErbB3 in breast cancer cells. Transl Res *207*: 44-55, 2019. PMID: 30639369. DOI: 10.1016/j.trsl.2018.12.007
21 Turczyk L, Kitowska K, Mieszkowska M, Mieczkowski K, Czaplinska D, Piasecka D, Kordek R, Skladanowski AC, Potemski P, Romanska HM and Sadej R: FGFR2-Driven Signaling Counteracts Tamoxifen Effect on ERα-Positive Breast Cancer Cells. Neoplasia *19(10)*: 791-804, 2017. PMID: 28869838. DOI: 10.1016/j.neo.2017.07.006
22 Orwick-Rydmark M, Arnold T and Linke D: The use of detergents to purify membrane proteins. Curr Protoc Protein Sci *84*: 4.8.1-4.8.35, 2016. PMID: 27038269. DOI: 10.1002/0471140864.ps0408s84
23 Liu S, Yang H, Wan L, Cai HW, Li SF, Li YP, Cheng JQ and Lu XF: Enhancement of cytotoxicity of antimicrobial peptide magainin II in tumor cells by bombesin-targeted delivery. Acta Pharmacol Sin *32(1)*: 79-88, 2011. PMID: 21131998. DOI: 10.1038/aps.2010.162
24 Park CB, Yi KS, Matsuzaki K, Kim MS and Kim SC: Structure-activity analysis of buforin II, a histone H2A-derived antimicrobial peptide: the proline hinge is responsible for the cell-penetrating ability of buforin II. Proc Natl Acad Sci USA *97(15)*: 8245-8250, 2000. PMID: 10890923. DOI: 10.1073/pnas.150518097
25 Wang L, Dong C, Li X, Han W and Su X: Anticancer potential of bioactive peptides from animal sources (Review). Oncol Rep *38(2)*: 637-651, 2017. PMID: 28677775. DOI: 10.3892/or.2017.5778
26 Li D, Wang W, Shi HS, Fu YJ, Chen X, Chen XC, Liu YT, Kan B and Wang YS: Gene therapy with beta-defensin 2 induces antitumor immunity and enhances local antitumor effects. Hum Gene Ther *25(1)*: 63-72, 2014. PMID: 24134464. DOI: 10.1089/hum.2013.161
27 Garcia AE, Osapay G, Tran PA, Yuan J and Selsted ME: Isolation, synthesis, and antimicrobial activities of naturally occurring theta-defensin isoforms from baboon leukocytes. Infect Immun *76(12)*: 5883-5891, 2008. PMID: 18852242. DOI: 10.1128/IAI.01100-08
28 Tomko RJ Jr and Hochstrasser M: Molecular architecture and assembly of the eukaryotic proteasome. Annu Rev Biochem *82*: 415-445, 2013. PMID: 23495936. DOI: 10.1146/annurev-biochem-060410-150257
29 Mehta A, Zhang L, Boufraqech M, Zhang Y, Patel D, Shen M and Kebebew E: Carfilzomib is an effective anticancer agent in anaplastic thyroid cancer. Endocr Relat Cancer *22(3)*: 319-329, 2015. PMID: 25972243. DOI: 10.1530/ERC-14-0510
30 Jonker PK, van Dam GM, Oosting SF, Kruijff S and Fehrmann RS: Identification of novel therapeutic targets in anaplastic thyroid carcinoma using functional genomic mRNA-profiling: Paving the way for new avenues? Surgery *161(1)*: 202-211, 2017. PMID: 27865593. DOI: 10.1016/j.surg.2016.06.064
31 Okumura T, Ikeda K, Ujihira T, Okamoto K, Horie-Inoue K, Takeda S and Inoue S: Proteasome 26S subunit PSMD1 regulates breast cancer cell growth through p53 protein degradation. J Biochem *163(1)*: 19-29, 2018. PMID: 28992264. DOI: 10.1093/jb/mvx053
32 Bao J, Jiang X, Zhu X, Dai G, Dou R, Liu X, Sheng H, Liang Z and Yu H: Clinical significance of ubiquilin 1 in gastric cancer. Medicine (Baltimore) *97(3)*: e9701, 2018. PMID: 29505024. DOI: 10.1097/MD.0000000000009701
33 Lee MH, Zhao R, Phan L and Yeung SC: Roles of COP9 signalosome in cancer. Cell Cycle *10(18)*: 3057-3066, 2011. PMID: 21876386. DOI: 10.4161/cc.10.18.17320
34 Tundo GR, Sbardella D, Ciaccio C, De Pascali S, Campanella V, Cozza P, Tarantino U, Coletta M, Fanizzi FP and Marini S: Effect of cisplatin on proteasome activity. J Inorg Biochem *153*: 253-258, 2015. PMID: 26387966. DOI: 10.1016/j.jinorgbio.2015.08.027
35 Dimitrakis P, Romay-Ogando MI, Timolati F, Suter TM and Zuppinger C: Effects of doxorubicin cancer therapy on autophagy and the ubiquitin-proteasome system in long-term cultured adult rat cardiomyocytes. Cell Tissue Res *350(2)*: 361-372, 2012. PMID: 22864983. DOI: 10.1007/s00441-012-1475-8
36 Wang H, Yu Y, Jiang Z, Cao WM, Wang Z, Dou J, Zhao Y, Cui Y and Zhang H: Next-generation proteasome inhibitor MLN9708 sensitizes breast cancer cells to doxorubicin-induced apoptosis. Sci Rep *6*: 26456, 2016. PMID: 27217076. DOI: 10.1038/srep26456

Received September 21, 2021
Revised October 17, 2021
Accepted October 19, 2021