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In silico and *in vitro* studies of cytotoxic activity of different peptides derived from vesicular stomatitis virus G protein

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ABSTRACT

Objective(s): This study aims at exploring cytotoxic activity of different peptides derived from VSVG protein against MCF-7 and MDA-MB-231 breast cancer cell lines and human embryonic kidney normal cell (HEK 293).

Materials and Methods: The ANTICP web server was used to predict anticancer peptides. The cytotoxic activity of peptides with high score (P26, P7) and low score (P19) was examined by MTT and DNA fragmentation assays.

Results: The results obtained from ANTICP web server demonstrated that 4 out of 48 peptides (P26, P7, P10, and P16) had anticancer activity. P26 and P7 peptides of these 4 peptides were detected to have high cytotoxic activity against MCF-7 cells with CC_{50} values of 98,280 µg/ml and MDA-MB231 cells with CC_{50} 100,550 µg/ml, respectively. In addition, the results showed that amino acid residues of these 4 peptides were located near fusion domain.

Conclusion: The results confirmed that P26 and P7 peptides might induce membrane damage and initiate apoptosis. The present study suggested that P26 and P7 peptides could be appropriate candidates for further studies as cytotoxic agents and modifications in the residue at positions 70-280 might potentially produce a more efficient VSVG protein in gene therapy.

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Introduction

The vesicular stomatitis virus protein G (VSVG) is a transmembran glycoprotein, which is involved in virus attachment to the specific receptor at the cell surface (1). This protein has been widely used to study therapeutic gene delivery (2). However, the major limitation of using VSVG in gene therapy is the toxicity of the protein in high concentrations for host cells (3). Some results demonstrated that expression of VSVG protein is toxic to most normal and tumor cells (4, 5). Therefore, we were concerned about the effect of different peptides isolated from VSVG on cancer and normal cell lines. Several computational methods are available for predicting anticancer peptides. These methods facilitate designing therapeutic peptides with high toxicity against cancer cell lines. They are usually based on machine learning methods (6). Machine learning method can predict anticancer peptides using three different algorithm including Artificial Neural Network (ANN), Quantitative Matrices (QM) and Support Vector Machine (SVM). SVM model is a powerful algorithm, which is developed based on amino acid composition and binary profile features (7, 8). ANTICP is a web server which determines anticancer peptides based on SVM method. In the present study, SVM was used to predict peptides with high and low toxicity (9). Cytotoxic Activity of two peptides with high scores and one peptide with low score was studied against MCF-7 and MDA-MB-231 breast cancer cell lines and Human Embryonic Kidney normal cell line (HEK293).

Material and Methods

In Silco prediction

Dataset

First, amino acid sequences of VSVG protein were retrieved from NCBI web page (http: //www.ncbi.nlm.gov/protein). The VSVG protein sequence was divided into 48 overlapping peptides – each peptide consisted of 20 amino acids in length.

ANTICP tool

The anticancer activity of all VSVG peptides derived was predicted by adopting ANTICP web server computer program (crdd.osdd.net/ raghava/ Anticp). SVM methods were applied to predict and classify anticancer and non-anti cancer peptides.

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Prot param tool

ProtParam is a tool which calculates various physical and chemical parameters of a protein (10). This tool is available at (http://web.expasy.org/protparam). Four characteristics including Instability, PI, Hydropathicity and Aliphatic index of 4 positive peptides (Cytotoxic) and 44 negative peptides (Non cytotoxic) of VSVG were evaluated using ProtParam.

Statistical analysis using ROC curve

The data were analyzed using Receiver Operating Characteristic (ROC) analysis. ROC curve is a tool for organizing classifiers and visualizing their performance (11, 12). Statistical analysis of ROC curves was carried out by the STAR server (http://protein.bio.puc.cl/star/home.php) (13). The results displayed classification accuracy (ACC) and Area under curve (AUC).When ACC value is more than 80%, it indicates a significant difference between two classes.

Peptides preparation

Two peptides with high score and as well as one peptide with low score were purchased as synthetic peptides from China Shine gene company with purity of > 75% and used without further purification. The peptides were stored at -4˚C until they were used.

Culture medium and cell lines

MCF-7 and MDA-MB-231 breast cancer and HEK cell line were purchased from National Cell Bank of Pasture Institute, Tehran, Iran. Cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100U/ml penicillin and 100 µg/ml- streptomycin and 5Mm L-glutamine. The cell lines were cultured at 37˚C fewer than 5% CO_2 condition in a humidified atmosphere. All reagents and cell culture media were purchased from Gibco Company Germany.

Cytotoxicity assay

The cytotoxicity of peptides P26 and P7 with high and P19 with low cytotoxic activity of isolated VSVG protein was examined applying MTT assay (14). The peptides were initially dissolved in deionized water and then diluted to prepare working concentrations of 1000, 100, 10 and 1 µg/ml. The cells were grown in 96-well plates at a density of 5×10^4 cells per well. After incubation for 4 hr, the cells were treated with different concentrations of samples and incubated for 72 hr. Then, 25 µl of MTT solution (5 mg/ml) was added to each well, and the plate was re-incubated for 4 hr. Finally, the medium was removed and 100 µl of DMSO was added to solubilize the formazan crystals. The amount of formazan crystal was determined by measuring the absorbance at 492 nm using a micro plate spectrophotometer (Awareness Technology Inc Stat fax 2100). All assays were carried out in triplicate.

DNA fragmentation assay

The potential cytotoxic activity of P26 and P7 peptides against breast cell lines was studied by DNA fragmentation assay (15). DNA fragmentation assay is a hallmark of apoptosis in many cell types. The 2×10^6 cells per ml were incubated with two kinds of peptides at CC_{50} concentration for 48 h. After stimulation, the cells were washed twice with Phosphate Buffer Solution (PBS). DNA was purified from the cells with High Pure Nucleic acid Kit (Roche, USA) according to the standard protocol. Purified DNA was re-suspended in loading dye (Fermentas R0611) and run on 1.8 % agarose gel in 1X TAE buffer. DNA fragmentation was visualized under UV transilluminator (Uvitec, England).

Statistical analysis

Data from three independent experiments were presented as mean ± SD. The CC_{50} value was calculated by Microsoft Excel 2012. One way ANOVA was used, followed by a *post-hoc* test (DUNCAN) and repeated measure ANOVA test. *P value* of ≤0.05 was considered as the measure of statistical significance between samples.

Results

ANTICP analysis

The results showed that P26, P7, P10 and P16 peptides had scores more than 0.9 (90%) and were represented as anticancer peptides. Other peptides with a score lower than 0.9 were determined as non-anticancer peptides. P26 and P7 peptides with higher scores and P19 with a lower score were selected respectively as positive (cytotoxic) and (non cytotoxic) data set for *in vitro* experiments (Table 1).

Analysis of protParam results

The ACC values among 4 anticancer peptides (P26, P7, P10 and P16) and 44 non-anti cancer peptides obtained from ROC analysis are indexed in Table 2. The results of ROC analysis showed that instability and aliphatic index between these two groups were significantly different.

Cytotoxicity assay

Different concentrations of P26, P7 and P19 (1, 10,100.1000) were tested for cytotoxicity against MCF7, MDA-MB-231 and HEK cell lines (Figure 1). The results indicated that both cytotoxic peptides inhibited viability of MCF-7 and MDA-MB-231 cell lines in a dose-dependent manner. The CC_{50} values of P26 and P7 peptides were estimated 78 and 280 µg/ml for MCF-7 cells, 100 and 550 µg/ml for MDA-MB 231 cell, respectively. The results of one way ANOVA and repeated measure ANOVA indicated that

Table 1. Amino acid sequences of peptides corresponding to VSVG protein

Peptide ID	Amino acid	Peptide sequence	SVM score	Prediction
P1	1-20	MKCLLYLAFLFIGVNCKFTI	0.78	Non-Anticp
P2	10-30	FIGVNCKFTIVFPHNQKGNW	0.86	Non-Anticp
P3	20-40	VFPHNQKGNWKNVPSNYHYC	0.68	Non-Anticp
P4	40-60	PSSSDLNWHNDLIGTALQVK	0.79	Non-Anticp
P5	50-70	DLIGTALQVKMPKSHKAIQA	0.73	Non-Anticp
P6	60-80	MPKSHKAIQADGWMCHASKW	0.75	Non-Anticp
P7	70-90	DGWMCHASKWVTTCDFRWYG	0.95	Anticp
P8	80-100	VTTCDFRWYGPKYITHSIRS	0.66	Non-Anticp
P9	90-110	PKYITHSIRSFTPSVEQCKE	0.75	Non-Anticp
P10	100-120	FTPSVEQCKESIEQTKQGTW	0.93	Anticp
P11	110-130	SIEQTKQGTWLNPGFPPQSC	0.66	Non-Anticp
P12	120-140	LNPGFPPQSCGYATVTDAEA	0.77	Non-Anticp
P13	130-150	GYATVTDAEAVIVQVTPHHV	0.44	Non-Anticp
P14	140-160	VIVQVTPHHVLVDEYTGEWV	0.64	Non-Anticp
P15	150-170	LVDEYTGEWVDSQFINGKCS	0.78	Non-Anticp
P16	160-180	DSQFINGKCSNYICPTVHNS	0.90	Anticp
P17	170-190	NYICPTVHNSTTWHSDYKVK	0.70	Non-Anticp
P18	180-200	TTWHSDYKVKGLCDSNLISM	0.83	Non-Anticp
P19	190-210	GLCDSNLISMDITFFSEDGE	0.43	Non-Anticp
P20	200-220	DITFFSEDGELSSLGKEGTG	0.70	Non-Anticp
P21	210-230	LSSLGKEGTGFRSNYFAYET	0.78	Non-Anticp
P22	220-240	FRSNYFAYETGGKACKMQYC	0.80	Non-Anticp
P23	230-250	GGKACKMQYCKHWGVRLPSG	0.55	Non-Anticp
P24	240-260	KHWGVRLPSGVWFEMADKDL	0.71	Non-Anticp
P25	250-270	VWFEMADKDLFAAARFPECP	0.63	Non-Anticp
P26	260-280	FAAARFPECPEGSSISAPSQ	0.97	Anticp
P27	270-290	EGSSISAPSQTSVDVSLIQ	0.73	Non-Anticp
P28	280-300	TSVDVSLIQDVERILDYSLC	0.74	Non-Anticp
P29	290-310	VERILDYSLCQETWSKIRAG	0.88	Non-Anticp
P30	300-320	QETWSKIRAGLPISPVDLSY	0.65	Non-Anticp
P31	310-330	LPISPVDLSYLAPKNPGTGP	0.73	Non-Anticp
P32	320-340	LAPKNPGTGPAFTIINGTLK	0.77	Non-Anticp
P33	330-350	AFTIINGTLKYFETRYIRVD	0.82	Non-Anticp
P34	340-360	YFETRYIRVDIAAPILSRMV	0.71	Non-Anticp
P35	350-370	IAAPILSRMVGMISGTTTER	0.84	Non-Anticp
P36	360-380	GMISGTTTERELWDDWAPYE	0.44	Non-Anticp
P37	370-390	ELWDDWAPYEDVEIGPNGVL	0.67	Non-Anticp
P38	380-400	DVEIGPNGVLRTSSGYKFPL	0.59	Non-Anticp
P39	390-410	RTSSGYKFPLYMIGHGMLDS	0.67	Non-Anticp
P40	400-420	YMIGHGMLDSDLHLSSKAQV	0.70	Non-Anticp
P41	430-450	SQLPDDESLFFGDTGLSKNP	0.82	Non-Anticp
P42	440-460	FGDTGLSKNPIELVEGWFSS	0.81	Non-Anticp
P43	450-470	IELVEGWFSSWKSSIASFFF	0.87	Non-Anticp
P45	460-480	WKSSIASFFFIIGLIIGLFL	0.68	Non-Anticp
P46	470-490	IIGLIIGLFLVLRVGIHLCI	0.78	Non-Anticp
P47	480-500	VLRVGIHLCIKLKHTKKRQI	0.83	Non-Anticp
P48	490-510	KLKHTKKRQIYTDIEMNRLG	0.85	Non-Anticp

cytotoxic activity of P26 and P7against MCF and MDA-MB 231 cells was significantly more than P19 ($P \leq 0.05$). Furthermore, the results showed that cytotoxic activity of P26 against MCF7 was statically more than MDA-MB231 cells ($P \leq 0.05$). Peptides (P26, P7) had no toxic effect on normal cells and no significant difference was found between the peptide (P19) and peptides P26 and P7 ($P \geq 0.05$).

Induction of apoptosis

MCF7 and MDA-MB 231 cells treated with P26 and P7 peptides at CC_{50} concentration indicated the presence of DNA fragmentation which confirmed anti-proliferative effect of these peptides (Figure 2). But both cells treated with P19 peptide did not provide any fragmentation. The result also demonstrated that HEK cells treated with these three peptides did not show any DNA fragmentation.

Table 2. The ACC (Accuracy) values of ProtParam results

Classifier	P1-4	P4-8	P8-12	P12-16	P16-20	P20-24	P24-28	P28-32	P32-36	P36-40	P40-44
Aliphatic	0.87	0.75	1	0.87	0.87	0.87	1	0.87	1	0.87	0.87
Instability	0.87	0.75	0.87	0.75	0.87	0.87	0.87	0.87	0.87	0.75	1
Hydrophaty	0.75	0.75	0.75	0.75	0.75	0.62	0.87	0.87	0.87	0.62	0.75
pI	0.75	0.87	0.75	0.75	0.62	0.75	0.75	0.75	0.75	0.75	0.62

Breakdown of DNA molecule is a sign of inhibition of DNA replication, which may be due to the inhibition of topoisomerase, key enzyme in DNA replication.

Discussion

In this study, cytotoxic activity of different peptides isolated from VSVG protein has been investigated. The stable expression of VSVG protein was reported to be toxic to most normal and cancer cells (16). Therefore, the use such protein in gene therapy has been limited (17). In the present study, P26, P7, P10 and P16 peptides derived from VSVG are predicted as anti cancer peptides using crdd.osdd.net/ raghava /Anticp. These peptides may be responsible for cytotoxic activity of this protein. Two out of four high score peptides (P26 and P7) were tested on breast cancer cell lines. These peptides showed potent cytotoxic activity against MCF7 and MDA-MB231 cells. Recently, anti cancer peptides have been verified as good candidates for anticancer drugs. Some peptides such as BMAP-27, Gaegurin 5 and 6 indicated cytotoxicity against various human leukemia cell lines and breast carcinoma cells (18). Peptides were reported to have the potential to kill cancer cells via electrostatic interactions between cell membrane component and the peptide; moreover, peptides could stimulate apoptosis in cancer cells via mitochondrial membrane interference of subsequent uptake peptides into cytoplasm (19). The results of the current study demonstrated that cytotoxic activity of these peptides against MCF7 and MDA-MB 231 cells was significantly more than that of the normal cells ($P \leq 0.05$). This may be related to differences in the membrane composition of cancer cells and normal cells. Previous research has shown that factors such

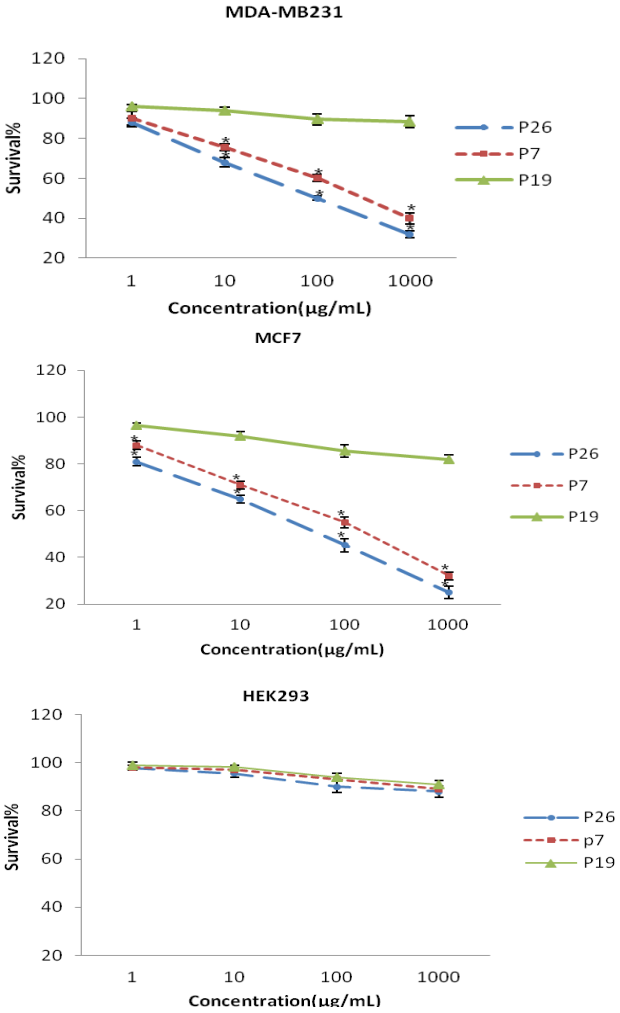

Figure 1. Cytotoxic activity of P26 (▬●▬) P7 (▪▪▪■▪▪▪) and P19 (▬▲▬) peptides against MDA-MB231- MCF7, and HEK cell lines. Cells were treated with different concentrations of peptides for 72 hr. The effect was measured by MTT cell viability assay. The result is representative of means ± SD of three independent experiments.*$P \leq 0.05$ compared to non anticancer peptide (P19)

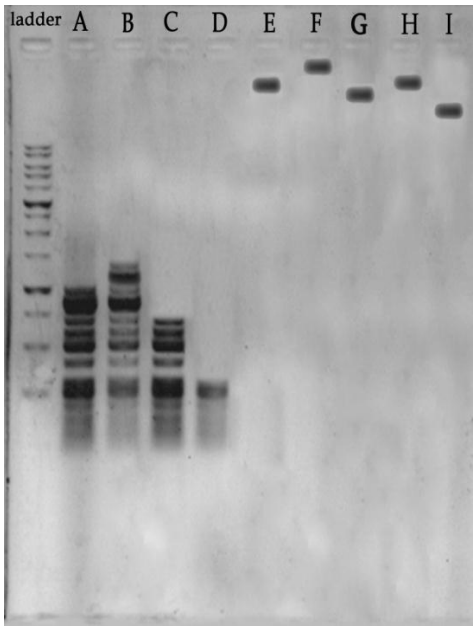

Figure 2. MDA-MB-231, MCF7 cells treated with P7, P26 (Cytotoxic), and P19 (Non cytotoxic) at CC_{50} concentration and HEK cells were treated with 1000 µg/ml of P26, P7 and P19 for 48 hr. Ladder: 100 bp DNA. MCF7 treated with P7 (A), P26 (B), P19 (F). MDA-MB-231 treated with P26 (C), P7 (D), P19 (E). HEK treated with P19 (G), P7 (H), P26 (I)

as differences in cell membrane composition, fluidity and numbers of micro villi between cancer cells and normalones may be the explanation for the ability of certain peptides to kill cancer cells (20). Instability and aliphatic properties were significantly different between 4 anticancer and 44 non-anticancer peptides. Previous studies demonstrated that a number of factors including hydrophobicity, amphipathicity and instability could be important for cytotoxic activity (21). Aliphatic index has been regarded as a positive factor for the increase of cytotoxicity against cells and viruses (22). Aliphatic index is generally defined by aliphatic side chain (alanine, valine, isoleucine and leucine) of proteins (23). According to the findings of this study, Cytotoxic peptides P26, P7, P10 and P16 have been located between residues, 260-280, 70-90, 100-120 and 160-180, respectively. The previous results showed that the region between residues 59-221 of VSVG protein was close to the membrane during interaction with the host cell membrane (24). Further, previous experiments have demonstrated that affinity of the VSVG protein for cancer cells was significantly more than normal cells (25). The results suggested that cytotoxic activity of VSVG peptides possibly had relevance to the context of the cancer cell receptors. The conclusion drawn is that four presented cytotoxic peptides were located near the fusion domain and may induce membrane damage and apoptosis through the death receptors pathway. This finding demonstrates that the changes in the residue at position 70-280 are essential to obtain nontoxic VSVG protein.

Conclusion

The results indicated that P26 and P7 peptides could be appropriate candidates for *in vivo* testing as cytotoxic agent. The changes in this protein are also crucial to decrease cytotoxicity of it.

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References

1. Seganti L, Superti F, Girmenia C, Melucci L, Orsi N. Study of receptorsfor vesicular stomatitis virus in vertebrate and invertebrate cells. Microbiologica 1986; 9:259–267.

2. Schauber CA, Tuerk MJ, Pacheco CD, Escarp PA. Lentiviral vectors pseudo typed with baculovirus gp64 efficiently transduce mouse cells *in vivo* and show tropism restriction against hematopoietic cell types *in vitro*. Gene Ther 2004; 11:266–275.

3. Molina RP, Hongqing Q-Ye, Brady J, Christine A, Michael Kaleko M, LuoT. Baculovirus GP64 pseudo typed bovine immunodeficiency. Mol Ther 2004; 9: S2779.

4. Ory DS, Neugeboren BA, Mulligan R. A stable human-derived packaging cell line for production of high titer retrovirus vesicular stomatitis virus G pseudo types. Proc Natl Accad Sci USA 1996; 93: 11400–11406.

5. Qiao J. Moreno J, Forshaw M, Rosa M, Diaz and Richard G. VSV-G pseudo typed MLV- based, semi-replication-competent retroviru.ses for cancer gene therapy. Mol Ther 2004; 9:S282–S282.

6. Sharma A, Kapoor P, Gautam A, Chaudhary K, Kumar R. Computational approach for designing tumor homing peptides. Sci Rep 2013; 3:1607.

7. Gupta S, Kapoor P, Chaudhary K, Gautam A, Kumar R. In silico approach for predicting toxicity of peptides and proteins. PLoS One 2013; 8:739 -757.

8. Michalski SR, Carbonell GJ, Mitchell MT. Machine learning: an artificial intelligence approach. Tioga Publishing Co1983; 1:463-482.

9. Tyagi A, Kapoor P, Kumar R, Chaudhary K, Gautam A , Raghava GPS. In silico models for designing and discovering novel anticancer peptides. Sci Rep 2013; 3:2984.

10. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, *et al*. Protein Identification and Analysis tools on the EXPASY Serv. the Proteomics Protocols Handbook. Humana Press; 2005. p.571-607.

11. Zadrozny B, Elkan C. Obtaining calibrated probability estimates from decision trees and naive Bayesian classier. In: Proc. Eighteenth Internat. Conf. on Machine Learning 2001; pp 609-616.

12. Hanley JA, McNeil BJ. The meaning and use of the area under receiver operating characteristic (ROC) curve. Radiology 1982; 143: 29-36

13. Fawcett T. An introduction to ROC analysis. Pattern Recognit Lett 2006; 27:861–874.

14. Meerloo van J, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. Methods Mol Biol 2011; 731:237-245.

15. Cotter TG, L**e**nnon SV, Glynn JG, Martin SJ. Cell death via apoptosis and its relationship to growth, development and differentiation of both tumor and normal cells. Anticancer Res 1990; 10:1153–1159.

16. Miyanohara A. Preparation of Vesicular Stomatitis Virus-G (VSVG) Conjugate and its Use in Gene transfer. Delivery and Expression of DNA and RNA. Cold Spring Harbor; 2012.

17. ShinTC, Akihiro L, Lin G, Theodore F, Jiinc-K Y. Generation of packaging cell lines for pseudo typed retroviral vectors of the G protein of vesicular stomatitis virus by using a modified tetracycline inducible system. Biochemistry 1996; 93:10057- 10062.

18. Hoskin DW, Ramamoorthy A. Studies on anticancer activities of antimicrobial peptides. Biochim Biophys 2008; 1778:357–375.

19. Zachowski A. Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. Biochem J 1993; 294:1–14.

20. Domagala W, Koss LG. Surface configuration of human tumor cells obtained by fine needle aspiration biopsy. Scan Electron Microsc 1980; 1:101–108.

21. Huang YB, Wang XF, Wang H, Liu Y, Chen Y. Studies on mechanism of action of anticancer peptides by modulation of hydrophobicity within a

defined structural framework. molecular cancer therapeutics 2011; 10:416-426.
22. Chang KY, Yang J-R. Analysis and poredictionof highly effective antiviral peptide based on random forests. PLoS One 2013; 8:e701.
23. Viader-Salvadó JM, Gallegos-Lopez JA, Gerardo Carreón-Trevino JG, Castillo-Galvan M, Roio- Dominguez A, Guerrero-Olazarán M. Design of thermo stable beta-propeller phytases with activity over a broad range of pHs and their overproduction by pichia pastoris. Appl Environ Microbial 2010; 19:6423- 6430.
24. Sun XI, Belouzard S, Whittaker GR. Molecular architecture of the bipartite fusion loops of vesicular stomatitis virus glycoprotein G, a Class III viral fusion protein. J Biol Chem 2008; 283:6418–6427.
25. Gaspar D, Salomé Veiga A, Miguel AR, Castanho B. From antimicrobial to anticancer peptide. Front Microbial 2013; 4:294.