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Design, Synthesis and Biological evaluation of Non-Hemolytic Membrane Disruptive Anticancer Peptides

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Abstract
Cancer is a disease in which abnormal cells of the body grow uncontrollably ultimately causing harm to the body. The property of contact inhibition is absent in cancerous cells and thus they proliferate disorderly giving rise to tumors. In this present study, we found that peptide 4 showed specific cytotoxicity to tumor cells. Further investigations revealed that Peptide 4 is capable of penetrating into cytoplasm and triggering cytochrome C release from mitochondria, which ultimately resulted in apoptosis. Meanwhile, Peptide 4 inhibited the migration of cancer cells. In conclusion, this peptide might be promising candidates for oncotherapy.

Keywords: **Anti-cancer peptides (ACPs), Anti-Microbial peptides (AMPs), Drug resistance-reversing effects, Membrane-disruption Peptides**

INTRODUCTION

Cancer comprises abnormal growth of cells in the body caused by a defect in the natural cellular systems that control cell division and reproduction.[1] It can occur in any part of the body, leading to the old cells not dying and growing, forming abnormal cells. This results in the formation of masses of tissue known as tumors.[2] Malignant tumors can spread to other body parts by transporting some cells from the tumor site to distant parts through the blood circulation or lymphatic system.[3,4] When new tumors form in other body areas, this is known as the spread of cancer or metastasis. Thus Cancer is consider as chronic disease, remains a one of the major human health problems. The number of deaths due to cancer can be reduced if the disease is detected at an early stage and suitable treatment begins immediately.[5] Standard treatments available for curing cancer includes surgery, Radiotherapy, Chemotherapy, Gene therapy, Hormone therapy, Bone marrow transplant, Cryosurgery, Immunotherapy, Photodynamic therapy, Peripheral stemcell transplant adopted alone or in combination.[6-9] However, these methods have their own limitations in their effectiveness. Surgery treatment is an effective approach only in the early stages or when the cancer is still localized.[10] Radiotherapy used in combination with surgery, is too expensive and long-lasting non-specific approaches that inevitably also damage healthy cells.[11] Chemotherapy is the use of drugs to inhibit or kill proliferating cancer cells while leaving host cells unharmed, or at least recoverable. Long-term use of chemoteraphy induces chemoresistance, which consists of theinnate and/or acquired capability of cancer cells to evade the effects of chemotherapeutics, making the treatment ineffective.[12] Consequently, there is an ever-increasing need to test and develop new therapeutic approaches for cancer treatment to overcome the limitations of conventional methods.

In latest years, therapeutic peptides have been attracting great interest in cancer therapy.[13,14] Anticancer peptides (ACPs) represent prominent anticancer therapeutic agents due to their high activity, great specificity, lower toxicity, rare side effect, slow immunogenicity, good biocompatibility and easy synthesis.[15-17] Various mechanisms for their anticancer property have been documented, including apoptosis induction[18,19], membrane disruption[20], DNA damage[21], angiogenesis inhibition [22], immunomodulation [23], and modulation of pathways involved in cell survival and proliferation[24]. Selectivity and mode of action of AMPs and ACPs seem to be similar in theory because a net negative charge is found on bacterial, as well as on tumor, cell surfaces.[25-28] However, many membranolytic ACPs suffer from toxicity towards non-cancer and red blood cells. Therefore, the design of new selective non-hemolytic anticancer peptides is of great interest. In the present work, we studied the selectivity potential of small hybrid peptides exhibiting inhibition of tumor growth by membranolytic action towards different cancer cell lines.

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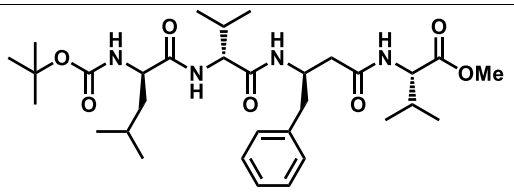
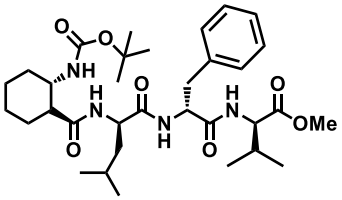
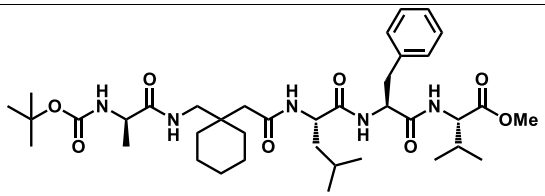
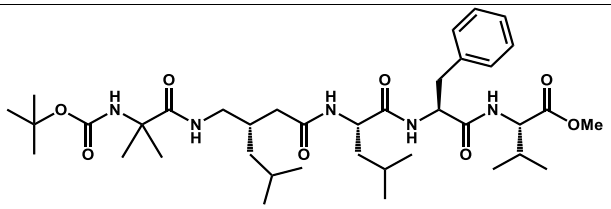
RESULTS AND DISCUSSION

The α-helical-forming propensity and amphipathic nature amino acid-rich peptides have long been associated with their ability to penetrate microbial and cancer cell membranes.[25] Hence we synthesize the, amphiphilic α-helical peptide **1-4** (Figure 1). Hers we carefully design the sequence of peptides by selecting high α-helical-forming propensity amino acid residues. Also in order to improve the specificity towards cancer cells and reduce the specificity towards normal cells we avoid the inclusion of charge amino acid residue in the peptide sequence.

Entry	Peptide structure	Peptide name	Calculate M.weight	Observed M. weight
1		**1**	604.79	627.2 (M + Na)
2		**2**	616.80	639.3 (M + Na)
3		**3**	715.93	738.4 (M + Na)
4		**4**	717.95	740.3 (M + Na)

SYNTHESIS OF PEPTIDES

Linear peptides **1**–**4** were synthesized by following conventional tert-butyloxy carbonyl (Boc)-based solution phase peptide synthesis. After the completion of synthesis, the peptides were precipitated with cold diethyl ether, dried, and finally purified by RP-HPLC using a linear gradient of aqueous acetonitrile in the presence of TFA (0.1%, v/v) as ion pair reagent on a Jupiter C_{18} column.

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CONFORMATIONAL STUDY OF PEPTIDES

Most antimicrobial (AMPs) and anticancer peptides (ACPs) fold into membrane disruptive cationic amphiphilic α-helices. Circular dichroism (CD) has been extensively used to spectroscopically study the structure of biomolecules in solution.[26] To explore the conformation of peptides **1-4** in the solution, we have carried out the circular dichroism (CD) study. For this, the CD spectra were recorded with 5 μM concentration of each peptide in PB buffer at pH 7.4 in the presence of 5 mM dodecyl phosphocholine (DPC). The CD spectra of peptides **1–4** taken at 25 °C are shown in Figure 2. Peptides 3 and 4 show a minimum at 220.5 nm and 210 nm with a crossover at 195 nm which is characteristic of α-helix conformation. In contrast, the CD spectrum of peptides 1 and 2 are characterized by minima at 220 and crossover at 210 nm, suggesting that the peptides 1 and 2 does not fold in to α-helical conformation.

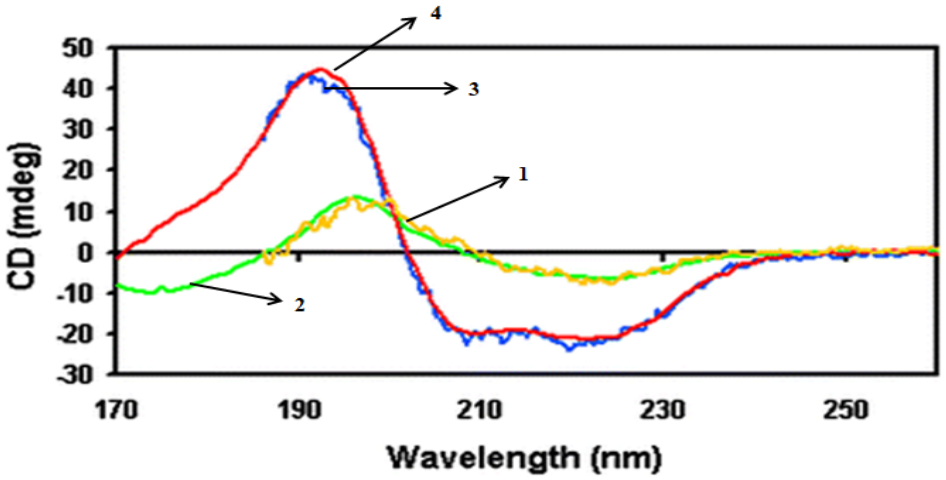

Figure 2: CD spectra of peptides 1- 4 taken in PB buffer at pH 7.4 and at 25 °C

Proteolytic degradation is considered as major weakness of peptide-based drugs, which limiting the systemic therapeutic applications of peptides. In order to produce maximum tumor-targeting effect, it is important that tumor-targeting peptides exhibit high metabolic stability in human plasma and reach intended target intact to deliver maximum tumor-targeting effect. The proteolytic degradation of all 4 peptides were investigated in vitro in human plasma. The metabolic stability of peptides **1-4** was determined by incubating each peptide with human plasma. The peptides (25μL) were incubated with human plasma (500 μL) in duplicate at 37 °C for up to 2 h. Following incubation at 1 and 2 h, the plasma proteins were precipitated with a mixture of CH_3CN/EtOH (1:1 v/v, 400 μL) and the sample was centrifuged (7000 rpm, 7 min). The supernatant layer was removed, filtered through Millex GP filter (0.22 μm), and analyzed by HPLC to determine the proteolytic stability of the peptides. The results of plasma stability studies of peptides **1-4** were represented in Figure 3. The estimated percent of peptides 3 and 4 remaining intact in plasma was found to be between 89 and 94% at 1 h and between 81 and 87% at 2 h, indicating a low enzymatic degradation of these two peptides. Peptide **1** and **2** might be degraded by some enzymes. Hence, peptides **3** and **4** are stable and suitable to target tumor tissue.

CELL PROLIFERATION AND VIABILITY ASSAY

Anti-cancer activity of the peptides **1-4** was assessed by the MTT -[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay. The assay was performed with several cell lines including normal HDF cells, Human liver cancer cell lines A549, human breast cancer cell lines MCF-7 and human colon carcinoma cell line HCT-116. As shown in figure 3, the peptides **3** and **4** with non-natural γ-amino acids at site 4 presented more favorable anti-cancer activities, while peptide **2** with all natural amino acids does not displayed anticancer activity. Interestingly peptide **1** with cyclic non-natural amino acid at N-terminal presented weakened anti-cancer effects. Additionally, all the peptides exhibited negligible toxicity to the normal HDF cell line.

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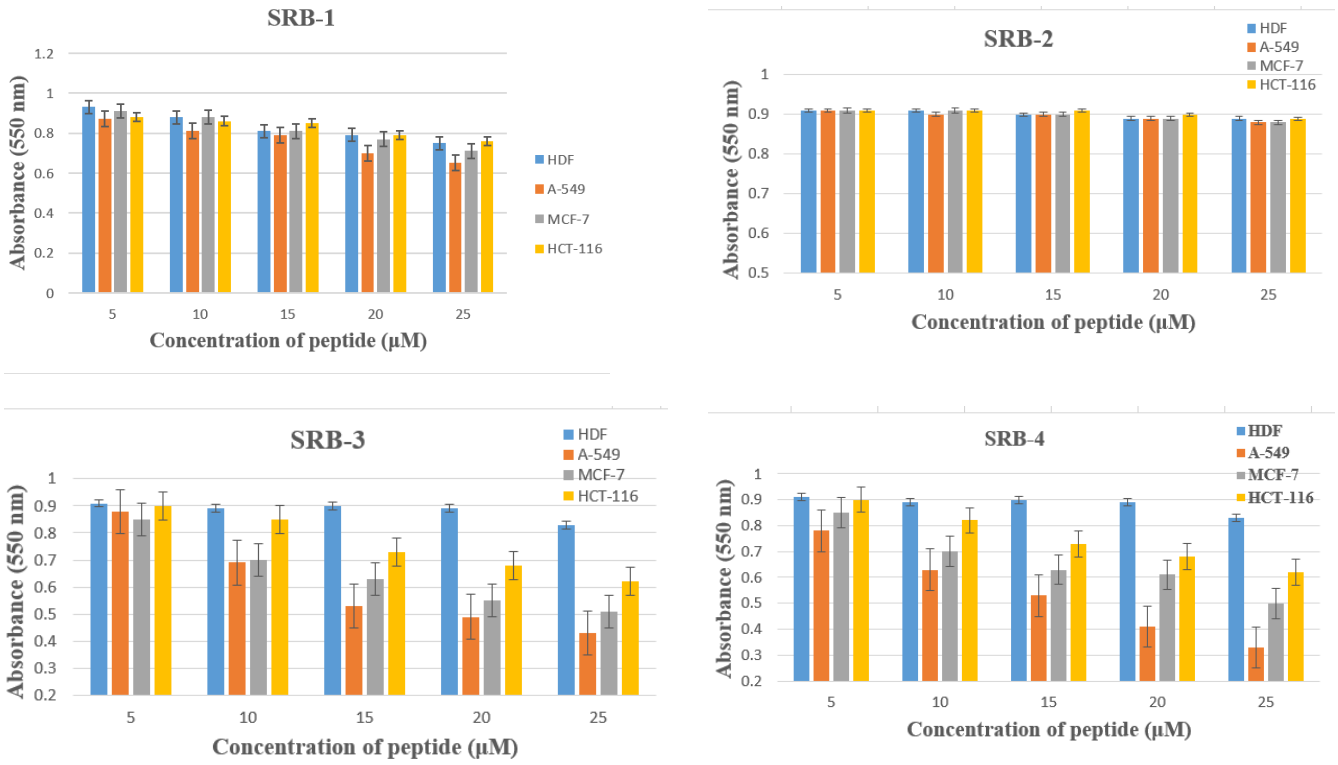

Figure 3: Inhibition of HDF, A549, MCF-7 and HCT-116 cells proliferation by peptides 1-4 assessed by 24 h MTT assay,

CELL MORPHOLOGY STUDY

To determine the morphological changes in cells, the A549 was treated with two different concentration of peptide 4. From examination of A549 cells morphology at 24 h after 10, and 25 µM peptide 4 treatment is shown in figure 3. It was observed that cells started shrinking showing the symptoms of the cell death. Further, it was clear that the total cell count started decreasing in the GA treated group and this effect was observed that this effect was observed in a concentration dependent mai

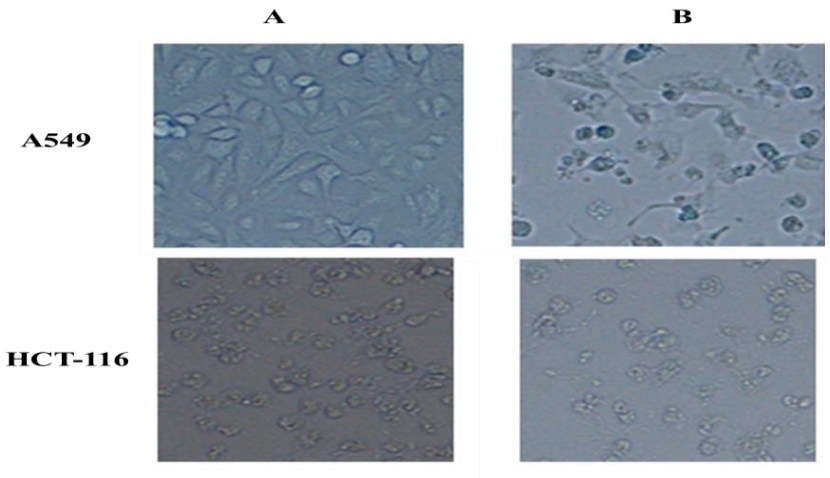

Figure 4: Morphology of A549 and HCT-116 cells treated A) without and B) with 17 µM of peptide 4.

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Wound healing assay

The inhibition of cell migration and metastases is an important consideration in cancer therapy. Angiogenesis can induce in cancer cells under the microenvironment in tumor tissue, which can migrate to other tissue from primary tissue. Hence a wound-healing assay was conducted to confirm that peptide 4 has an inhibitory capability on cell migration and metastases of A549 and HCT-116 cells. A549 and HCT-116 cells were treated with peptide 4 at a concentration of 17 μM, respectively. The result observed is shown in Figure 5. It is clear that, peptide 4 has an inhibitory capability on cancer migration of A549 and HCT-116 in the treated concentrations. This result suggests that potential therapeutic interventions might be obtained for the treatment of lung cancer cells including A549 and HCT-116 cells.

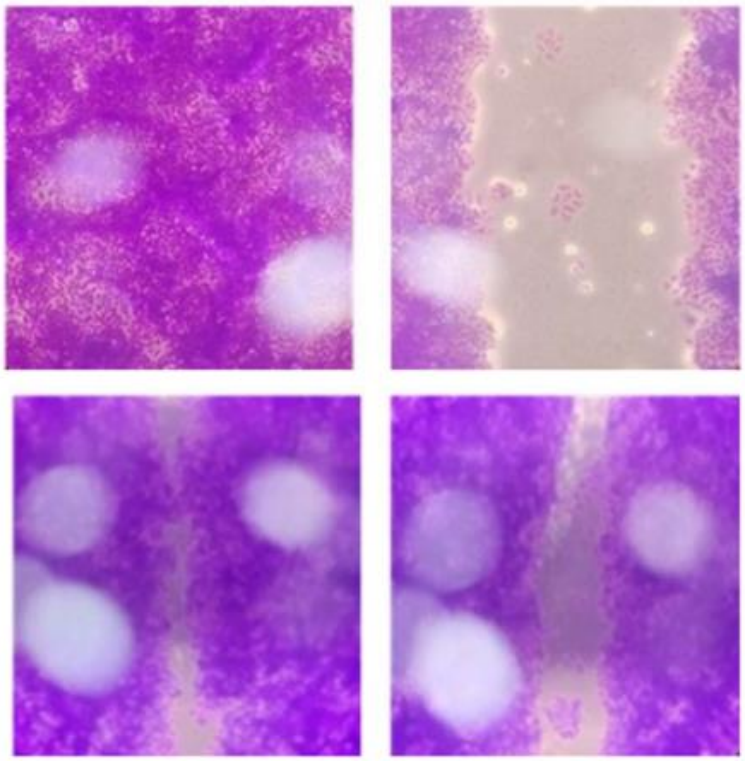

Figure. 5: A) Wound healing assay of human epithelial lung cancer cell lines. A Wound healing assay with A549 (control, left; peptide 4 treatment, right). B) Wound healing assay with HCT-116 (control, left; peptide 4 treatment, right). In both cases, peptide 4 had an extent of inhibition capacity for cancer cell migration.

GENERAL DISCUSSION

Developing peptide-based drugs that selectively inhibit cancer cells would establish efficient and reliable therapeuticsolutions.[51] Membranolytic ACPs have demonstrated broad-spectrum inhibition of cancer celllines and limited toxicity against normal cells. The present paper focused on evaluating the effects of peptides 1-4 on normal HDF cells, Human liver cancer cell lines A549, human breast cancer cell lines MCF-7 and human colon carcinoma cell line HCT-116. peptides constrained by γ-aminoacid residue promised a high proteolytic stability due to the inaccessibility of the peptide backbone to proteases. The cell validity assay with normal and different cancer cell lines, reveled that, none of the peptides were affected the cell viability of normal cell line HDF cells. Similarly peptide **1** is slightly affect the cell viabilities of A549, MCF-7 and HCT-116 cancer cell lines at higher concentration while peptide **2** is not affected both normal and cancer cell lines even at higher concentration. Cell viabilities of A549, MCF-7 and HCT-116 cells decreased with increasing concentration of peptides **3** and **4.** The cell viability decreased dose-dependently for A549 (lung cancer

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cell line) and MCF-7 cells. IC_{50} values were 13 μM and 17.3 μM for A549 and H460, respectively (Figure 3). This result shows that peptides **3** and **4** have an anti-cancer effect on lung cancer cell lines of A549, human colon carcinoma cell line HCT-116 and human breast cancer cell lines MCF-7 cells.
Further Peptides **3** and **4** inhibit A549 and HCT-116 cell proliferation in dose and time-dependent manner. Interestingly it is noticed that peptide **4** is more specific towards A549 lung cancer cell lines. Further from examination of A549 cells morphology at 24 h after peptide **4** treatment, it was observed that cells started shrinking showing the symptoms of the cell death but its effect on HCT-116 cell line is less significant compare to A549 cell line. Our result indicates that Overall, these results show that peptides **3** and **4** have a significant anti-cancer effect on the A549 lung cancer cell lines and MCF-7 human breast cancer cell lines.

CONCLUSION

In this work, we have demonstrated the selective properties of peptide **4** against A549 lung cancer cells. Cells morphological changes analysis of A549 cells revealed that peptide**-4** exhibit a membranolytic mode of action and kill the cell primarily by affecting the integrity of the cell membrane. The high sensitivity of A549 cells toward peptide-4 treatment can be attributed to the difference of the cell membrane composition. The extensive study of lipid imbalance in cancer cells can help to better understanding of mechanism of action which is beyond the scope of current work.

AUTHOR CONTRIBUTIONS:

Umashankara, M: Methodology, writing original draft preparation.

Spandana Rameshwar; Synthesis of peptide,formal analysis and conducting the biological analysis work.

Sunil kumar, Y.C. and Kumara, M. N. review the manuscript and editing,

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The institute had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results

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