Engineered Peptides for Targeting Breast Cancer Cells

by

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Abstract

Tumor targeting ligands provide a promising solution to the problems associated with the selective delivery of conventional cancer chemotherapeutics and diagnostics to cancer cells. Therefore, numerous cancer cell surface targeting peptides have been identified and evaluated. These peptides have shown specificity for cancer cells and promising results for tumor-selective drug delivery.

Kaurs group has reported on the development of analogues of p160 peptide that have shown increased affinity for breast cancer cell lines. An analogue of p160 (WxEAAYQkFL or 18-4DXK) when conjugated to doxorubicin showed specific targeting of breast tumors and demonstrated enhanced cytotoxicity against drug resistant cells as compared to free doxorubicin.

In this thesis, the design and synthesis of four analogues based on lead decapeptide 18-4DXK was performed and the interaction with breast cancer cells (MDA-MB-231, MDA-MB-435, and MCF-7) was evaluated. Binding with noncancerous cells (HUVEC and MCF-10A) was studied to identify the peptides that specifically bind to breast cancer cells over normal cells. The design strategy involved replacement of either D-norleucine (x) and/or D-lysine (k) amino acid in the lead sequence with the corresponding L residues or cyclization of the sequence.

The results show that cyclic (c18-4DK) peptide analogue displays higher (compared to other analogues) and more specific uptake by the breast cancer

cells. This third generation analogue of p160 (Cyclic 18-4DK) cancer targeting peptide is a better candidate peptide for conjugation to drugs or drug carriers for targeted drug delivery in breast cancer treatment.

Dedication

I would like to dedicate this thesis to my dear husband, Dharmendra for his practical and emotional support over the course of my research. I am truly thankful for having you in my life. My parents, for being the source of inspiration and encouragement throughout my life. Special thanks to my mom for her remarkable patience, love, support and for the countless days of Granny daycare to our beautiful son, Aryaman, during the final months of my research. Without their help, support and love this degree would not have been fulfilled.

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List of Abbreviations

BOP	l-Benzotriazol-1-yloxy-tris (dimethylamino)-phosphonium hexafluorophosphate
DCM	Dichloromethane
DMEM	Dulbecco's modified eagle medium
DMF	N,N-dimethylformamide
DNA	Deoxyribonucleic acid
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FMOC	9-Fluorenylmethyloxycarbonyl
FITC	Fluorescein-5- isothiocyanate
HCTU	(2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate)
RP-HPLC	Reversed phase High performance liquid chromatography
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
MEGM	Mammary epithelial cell growth medium
NMM	N-methyl morpholine
OBOC	One bead one compound
PEG	Polyethylene glycol
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane
WHO	World Health Organization

Amino acids Abbreviations

Ala (A)	Alanine
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartic acid
Cys (C)	Cysteine
Gln (Q)	Glutamine
Glu (E)	Glutamic acid
Gly (G)	Glycine
Leu (L)	Leucine
Lys (K)	Lysine
Phe (F)	Phenylalanine
Pro (P)	Proline
Tyr (Y)	Tyrosine
Val (V)	Valine
Nle(X)	Norleucine
β-Ala (Z)	β−Alanine

Chapter 1 Introduction

Breast cancer is the second leading cause of cancer related deaths in women after lung cancer. There were an estimated 508,000 deaths world-wide in the year 2011 according to estimates of World Health Organization (WHO). In 2014 alone Canadian cancer society estimated that 24,400 women and 210 men were diagnosed with breast cancer. These figures represent 26% of all new cancer cases in women in 2014. There were an estimated 5,000 deaths of women, which represent 14% of all cancer deaths in women in 2014 (http://www.cancer.ca/en/cancer-information/cancer-101/canadian-cancer-statistics-publication/?region=sk). Based on 2009 statistics 1 in 9 Canadian women are expected to develop breast cancer during her lifetime and 1 in 30 will die from it.

However, in the majority of developed countries, in spite of an increasing incidence, breast cancer mortality has been decreased owing to a better understanding of tumor biology, improved diagnostic devices and treatment specifically during the last decade.

1.1 Breast Cancer and Limitations of Current Treatment Options

Conventional clinical approaches include surgery, radiation and chemotherapy. Surgery and radiation are restricted to patients with localized and small size tumors and still the chances of relapse are very high. Surgery requires removal of partial or entire infected breast and some surrounding tissue but there are very

high chances that residual cancer cells are left out. If the lump is in proportion to the rest of the breast, mastectomy (removal of breast) is performed. Therefore, neoadjuvant therapy may be given before surgery to shrink the tumor size, or adjuvant therapy, usually radiation is given after surgery to destroy any cancer cells that remain in the breast area or nearby lymph nodes. Radiation therapy has its own side effects as it can harm healthy cells and can cause other complications as well. Chemotherapy is usually started after surgery and most of the chemotherapeutic agents inhibit cell division non-specifically and thus can also harm healthy proliferating cells of the body. The side effects of chemotherapeutic agents usually depend on the kind of drug combination regimen administered and their dose. The prolonged therapy with these agents sometimes leads to development of drug resistance (1).The non-specific distribution of anticancer drugs in healthy organs or tissues lead to severe, sometimes life threatening side effects and ultimately results in failure of the treatment. Doxorubicin, epirubicin, paclitaxel, docetaxel and cyclophosphamide are examples of some of the chemotherapeutics.

The limitations of the conventional cancer therapies can be circumvented with the understanding of cell signalling networks that are involved in cell survival, proliferation and differentiation. This knowledge can lead to the design of specifically targeting strategies and pave the way for cancer therapies (2).

1.2 **Tumor targeting ligands**

The toxic effects of chemotherapeutics could be limited to the intended tumor tissue if the drug could be preferentially directed to the tumor site. It would, therefore be desirable to target chemotherapeutic agents to desired target such as tumor vasculature. The distinct physiology of the tumor tissue and its microenvironment combined with the complexity of the biological system presents significant hurdles in site-specific delivery of chemotherapeutics. One way to accomplish this is the delivery of therapeutic drugs via cell surface targeted ligands that are specific for overexpressed or mutated markers or receptors present on tumor cells relative to physiologically normal cells (2-4). This increases the exposure of tumor cells to targeted therapeutics. The receptor mediated tumor targeting ligands include the use of proteins such as monoclonal antibodies, peptides, nucleic acids such as aptamers and other receptor ligands such as affibodies, growth factors, vitamins and carbohydrates. These ligands allow targeted delivery of drug of interest to the tumor site either by direct coupling **(Figure 1.1)** or through a carrier delivery system, such as liposomes, micelles and nanoparticles (5-8).

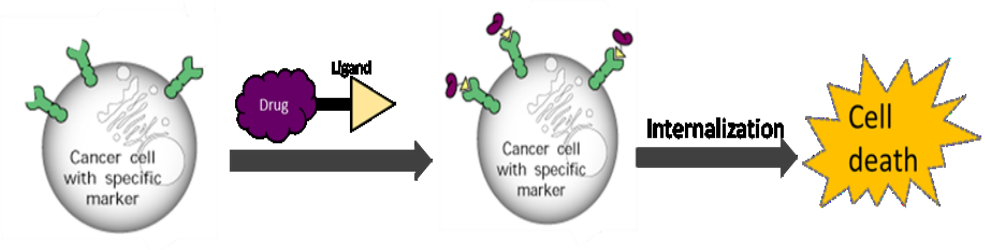


Figure 1.1 Schematic showing cancer cell targeting using drug-ligand conjugate.

The two different classes of tumor targeting ligands such as antibodies and peptides are discussed below.

1.2.1 Monoclonal antibody

Several studies have shown that antibodies and antibody fragments can improve tumor targeting (9, 10). Antibodies in native state and their recombinant fragments have been widely used for active drug targeting due to the specificity of the antigen-antibody interaction, as a result chemotherapeutic agents can be specifically delivered to the desired targeted site. Most monoclonal antibodies that are used for this purpose fall into the IgG class of immunoglobulins.

Monoclonal antibodies work as targeted therapies by 'targeting' specific proteins (receptors) on the surface of cells. Trastuzumab (Herceptin) is the first clinically approved humanized anti-HER2 monoclonal antibody for the treatment of breast cancer. It blocks the function of overexpressed HER-2 protein present on breast cancer cells (11). HER-2 positive breast cancer cells are more aggressive than other types of breast cancer cells (12). When combined with chemotherapeutics like doxorubicin and paclitaxel the trastuzumab (Herceptin) shows very good anticancer activity (13). Examples of other monoclonal antibody targeted therapies include cetuximab (anti-EGFR) and bevacizumab (anti-VEGF). The major limitations of antibody targeted therapy is the large size of the molecule (~160 KDa) which leads to poor tumor penetration and non-specific uptake by the liver and reticuloendothelial system because of the presence of Fc domain, mAbs

bind to normal cells and lead to immunogenicity (14, 15). These limitations results in dose limiting toxicity to normal tissues (16).

To overcome the size limitation of full length antibodies, a variety of antibody fragments i.e like antigen-binding fragments (Fab), dimers of antigen-binding fragments F.(ab′)^2, single-chain fragment variables (scFv) and other engineered fragments, have been developed (17, 18). They lack the Fc domain of the antibody which is responsible for non-specific binding of whole antibodies and thus show reduced nonspecific binding. The huge selectivity and ease of design and modifications has facilitated the use of antibody fragments in various applications like diagnostics, as biopharmaceutical drugs and delivery vehicles in gene therapy (19). The drawback of these antibody fragments is less stability and reduced antibody binding avidity (6, 20).

1.2.2 Peptides

Peptides alleviate some of the problems associated with antibody-targeted therapy. Some of them have binding affinities comparable to specific antibodies, despite their small size (21). In recent years, they have gained a lot of attention as cancer targeting ligands and offer tumor specific and less toxic alternative therapy by binding to specific markers on tumor cell surface. They have immense potential in the field of drug delivery because of their unique properties like small size, ease of synthesis and purify, safety in terms of immunogenicity, toxicity and pathogenicity and feasibility in conjugating to radionuclides, cytotoxic drugs or

toxins (22). Moreover, novel peptide analogs can be designed by chemical modifications for specific binding and metabolic stability.

Peptides can be used in targeted drug delivery as peptide drug conjugates, by conjugating to chemotherapeutics or as peptide nanocarrier conjugates by decorating the surface of drug delivery vehicles such as liposomes, micelles and nanoparticles carrying cytotoxic drugs (23-25).

Peptide drug conjugates offer many advantages over antibody drug conjugates. Owing to their small size, peptides are almost immunologically inert and cannot be recognized by the immune system therefore the conjugates reach the tumor site escaping the vasculature and p-glycoprotein efflux that causes drug resistance compared to the antibody drug conjugates. It is also possible to control the peptide drug ratio and chemistry of conjugation with the peptides. Examples include targeting of antitumor agent cytokine TNF by conjugating to RGD and NGR peptides. It was found that the conjugate was effective in 1000 fold lower dose than usual dose while reducing the toxic side effects (26).

Studies have found that peptide decorated nano carriers are more effective in the reduction of tumor growth compared to the non-targeted ones. For example, p160 decorated micelles show better uptake than c(RGDfK)-micelles and enhanced cytotoxicity of paclitaxel against MDA-MB-435 cancer cells (27). Peptides can also be used for in vivo optical fluorescence imaging by attaching them to dyes and fluorescent nanoparticles. They have also shown promising results in tumor imaging (28, 29).

A list of some of the approved peptide therapies for various applications are depicted in **(Table 1.1).** Despite, their enormous applicability in drug delivery, the inherent instability towards enzymes, difficulty to maintain secondary structure, and fast renal clearance restricted their use in some applications. The peptides need to be stabilized in order to reach the target and have intended effect.

Table 1.1: List of some of the FDA approved peptide-based therapeutics on the market.

Brand name	Generic name	Indication	Manufacturer
Bigonist®	Buserelin	Advanced prostate cancer	Sanofi-Aventis
Velcade®	Bortezomib	Multiple myeloma	Millennium Pharms, Janssen-Cilag
Zadaxin®	Thymalfasin	Chronic hepatitis B and C	SciClone Pharms International
Fuzeon®	Enfuvirtide	AIDS	Roche, Trimeris
Zoladex®	Goserelin	Breast and prostate cancer, endometriosis	AstraZeneca
Victoza®	Liraglutide	Type 2 diabetes	Novo Nordisk
Natrecor®	Nesiritide	Congestive heart failure	Scios
Sarenin®	Saralasin acetate	Hypertension	Norwich-Eaton Pharms, Procter & Gamble
Angiomax®	Bivalirudin	Angina	Medicines Company
Sandostatin®	Octreotide	Acromegaly, diarrhea	Novartis

1.3 **Methods used in the discovery of cancer targeting peptides**

The available research literature suggests that peptides are good alternatives for cancer drug targeting. However, the major challenge is to prepare and screen the peptide sequence with high affinity and specificity for cancer cells. The various strategies that can be used to discover cancer targeting peptides are: (i) Molecular modeling, if the x-ray structure of the receptor is known (ii) modifying the known ligands against cancer-associated receptors (bradykinin analogues against bradykinin receptor (30), and (iii) screening chemical or combinatorial library (31).

Screening combinatorial library is a great tool for basic research and have successfully been used by several investigators in the last few decades to discover cell surface binding peptides for a variety of target receptors. Since its discovery in the mid-80s, the field of combinatorial chemistry has advanced rapidly. Libraries of compounds can be generated by various techniques of combinatorial library and screened by a number of different assays. All combinatorial library techniques involve three main steps: (i) Library preparation, (ii) screening of the library components, and (iii) determination of the chemical structures of active compounds (32). The six different methods of combinatorial libraries for preparing and screening peptides are: (1) biological method phage display (33-35), (2) the OBOC (One bead one compound) combinatorial library method (32, 36). (3) Spatially addressable peptide library methods like Multi-pin technology, 96–well-plate libraries, Nanocan technology, SPOTs-membrane, chemical

microarray (37-39), (4) synthetic library methods requiring deconvolution like Iterative approach, Positional scanning, Recursive deconvolution approach, Orthogonal partition approach, Dual recursive deconvolution (39-41), (5) self-assembled peptide nucleic acid (PNA) encoded chemical microarrays (42), (6) the affinity selection method (43). Of all the above mentioned methods, only two approaches, Phage display method and the OBOC method have been applied for the discovery of tumor cell surface targeting ligands. Results from the preclinical studies have shown peptides to be very potential targeting agents (31).

1.3.1 Phage display

The phage-display peptide library is a very powerful technique, first developed by Smith and coworkers (44). In the phage-peptide display M13 phage is most widely used because of its high capacity for replication and the ability to receive large DNA inserts into its genome. A stretch of random double-stranded DNA (dsDNA) is inserted in the gene of phage that encodes pIII protein. The phage was used to infect bacterial cell to express/ display L-amino acid containing peptide on virion surface. In this way a phage display library was created by preparing a random mixture of phage clones with each displaying a single peptide on the surface.

The phage-display peptide library is screened against immobilized target proteins, intact cells or in vivo selection by intravenous injection in mouse. The bound phages are eluted with weak acid, amplified in Escherichia coli, and then re-panned a second time for better selection. Multiple copies of phage are thus

produced, and the encoding amino acid sequence on the phage is then DNA sequenced (**Figure 1.2**) Various sequences encoding specific amino acids can be inserted into the Phage genome to increase diversity. Linear and cyclic libraries can be constructed by changing cysteine coding sequences (45). The method enables the researcher to generate millions of different phages. Several cancer targeting peptides have been identified by phage display technique and many of them developed into therapeutics in clinical trials (46). However, the main limitation of this technique is that only L-amino acids can be incorporated into these libraries, and incorporation of unnatural amino acids or other organic building blocks into these libraries is not feasible.

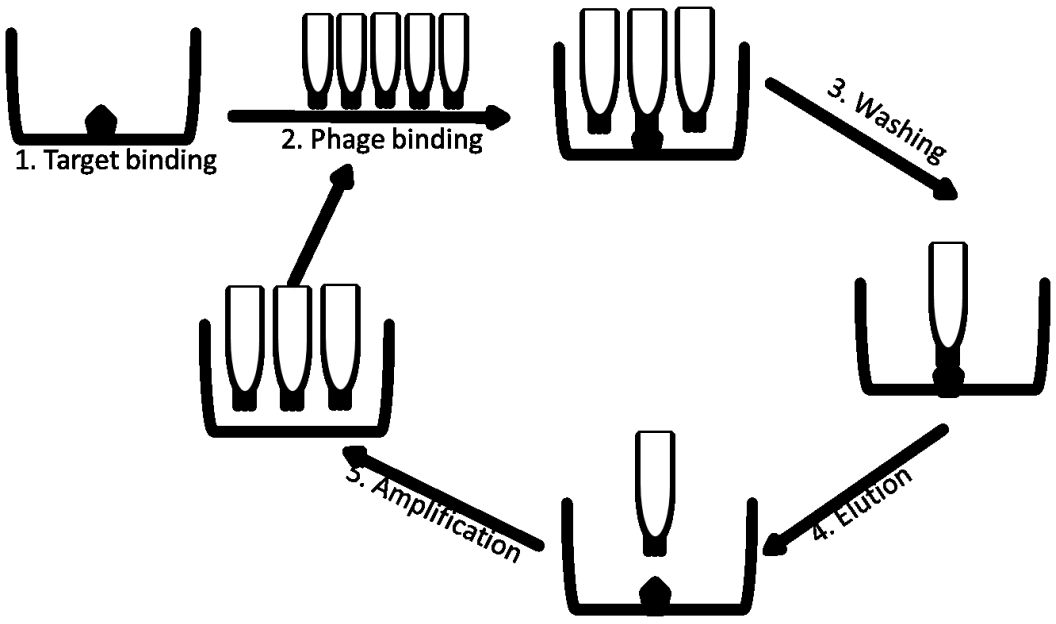


Figure 1.2: Affinity selection of phage-displayed peptide. 1. Target protein is immobilized. 2. Phage library is added. 3. Washing to remove unbound phage. 4. Elution of bound phage as a result of conformational changes to the binding site caused by pH change or other means which disrupts the interaction between displayed ligand and the target. 5. Amplification of eluted phage in E.coli for next round of bio panning.

1.3.2 One Bead One Compound Method (OBOC)

The one-bead one-compound (OBOC) library method was first recognized by Kit S. Lam group (36). It is based on the fact that combinatorial bead libraries can be prepared by a "split-mix synthesis" method, containing single polymer bead displaying only one type of entity, hence the name one-bead-one-compound combinatorial library method. Although chances are that there might be up to ten million copies of the same compound on a single bead. In this method, first the solid support is divided into 20 equal portions which is followed by coupling a different amino acid to each portion and then in the third step all portions are mixed together. These three steps comprise a cycle. Repeating the steps can elongate the length of chain and library can be generated. A target molecule is used to screen the library. This one-bead-one-compound library method was first successfully applied on over a million peptide beads to isolate peptide ligands that interact with anti-β-endorphin monoclonal antibody and streptavidin using an enzyme-linked colorimetric detection method (36). The main advantage of the OBOC method is that it does not require multistep synthesis and a large number of compounds can be synthesized and screened rapidly. One major disadvantage of the OBOC method is that each library compound is connected to the solid support via a linker, which may pose some steric hindrance between the cellular receptor and the library compound. However, the linker is beneficial in few cases (47).

1.4 **Chemical Modifications to enhance bioavailability of peptide therapeutics**

The application of peptides is limited for many therapeutic purposes mainly due to the rapid degradation by proteases and quick elimination from circulation via renal route. Unmodified peptides are prone to proteolysis and therefore do not circulate in the body for more than a few minutes. A variety of proteases are present in human plasma, including both exopeptidases and endopeptidases. Exopeptidases, aminopeptidases and carboxypeptidases in the gastrointestinal tract, break down sequences from the N- and C-termini, whereas endopeptidases act by cleaving the sites within amino acid sequences. The combination of these types of enzymes facilitates rapid and efficient degradation and is a significant barrier to the administration of peptide therapeutics via the gastrointestinal route.

Moreover, the small size leads to glomerular filtration in the kidney that can be prevented by increasing the molecular weight by attachment of macromolecules. Therefore, there is a need to perform chemical modifications that maximize the in vivo half-life of these bioactive peptides while preserving their selectivity.

Chemical modifications to the lead sequence can be done from relatively small modifications to more drastic modifications affecting the peptide backbone **(Figure 1.3).** The transformed peptide sequence obtained after modifying the backbone of the original sequence is called peptidomimetics. N- and C- termini protection/ modifications (48-51) to prevent from exopeptidases, alteration of peptide bond (52), incorporation of un-natural residues (53) to prevent from

endopeptidases, cyclization and conjugation to carrier moieties such as biocompatible polymers (54-57).

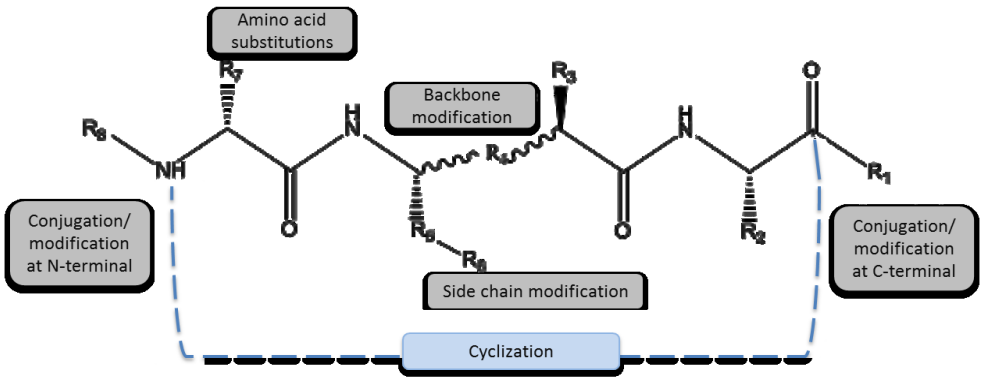


Figure 1.3: Examples of chemical peptide modifications. R_2, R_3, R_5, R_7 = Amino acid side chains, proteinogenic or otherwise. Possible modifications: R_1, R_8 = lipids, sugars, acetyls, polymers. R_4 = reduced, substituted peptidomimetic bonds. R_6 = methylation, acetylation, hydroxylation. R_7 = D-conformation, tetra-substituted amino acids.

1.4.1 N-terminal acetylation or C-terminal amidation

In serum or plasma, physiological degradation of peptides is required once they have exerted their function but it's a great challenge when they have been used therapeutically. Peptides are prone to enzymatic degradation, and have generally poor bioavailability in tissues and organs. They are degraded primarily by exopeptidases (amino- and carboxy peptidases) because of the free N- and C-termini. Therefore, end protection of the peptide is very important for its in vivo enzymatic stability. For example, the melanocyte-releasing hormone, MSH is naturally N-terminally acetylated and carboxy-amidated, and therefore inactivated by more specific endopeptidases (58). The in vivo half-life of the N-terminal

acetylated somatostatin analogue was improved from three minutes for the natural peptide to more than 400 minutes (59). Such improvement has also been obtained with pentapeptide analogues of a thymopoietin polypeptide involved in maturation of T lymphocytes. The half-life of the natural peptide was approximately one minute, whereas the double-protected analogue had no detectable degradation (60). Although end protection is widely used to improve enzymatic stability, it rarely prevents degradation completely. In most cases, it slows peptide metabolism, but degradation still occurs by the action of more specific endopeptidases.

1.4.2 Incorporation of Un-natural/non-proteogenic amino acids

Sometimes N-terminal acetylation may result in reduced biological activity because some peptides require free N-terminus for effective binding, therefore substitution of natural amino acids with the un-natural ones helps in greater enzymatic stability of drug candidate peptides (61). These substitutions can be: D-conformation, N-methylation, tetra-substitution, β-amino acids and side chain methylation(s) (62-64). For example, a D-peptide analogue of C-X-C chemokine receptor type 4 demonstrated dramatically improved stability. The natural L-peptide was degraded within 24 h, whereas the D-peptide showed no signs (53). Icatibant (Firazyr®, Shire), also contained unnatural amino acids which is a bradykinin B2 receptor antagonist decapeptide for the treatment of hereditary angioedema (65). The antimicrobial peptide heptaibin remained intact for 12 h with proteolytic enzyme, was also consisted of unnatural amino acids (66).

1.4.3 Cyclization

Cyclization of peptide sequences represents another widely used strategy to increase peptide potency, selectivity, stability and bioavailability. Cyclization confers a structural constraint, which reduces conformational flexibility and enhances selectivity and affinity for binding. Depending on functional groups, different strategies can be utilized for cyclization: for example, peptides can be cyclized head-to-tail (amino- and carboxy-groups of the N- and C-termini) head/tail-to-side-chain or side-chain-to-side-chain (67-69). The last two approaches allow selection of the amino acid used to form the linkage and usually residues, which are least important for activity are selected for this purpose. Another possibility for cyclic peptide formation is the formation of a disulfide bridge between two cysteine residues at the two termini (70). Furthermore, a ring can be established by a thioether link between the side chain of a C-terminally added cysteine residue and the α-carbon atom of an N-terminal acetyl group (71). Upon cyclization, a peptide ligand and its receptor showed higher affinity under structural constraint and reduced enzymatic degradation (72, 73). The reduced flexibility of the cyclic structures often would further insight into the development of optimized conformations for receptor targeting. One of the examples of cyclic polypeptide drug is bacitracin, used for intramuscular treatment of infants with staphylococcal pneumonia.

Cyclization of peptides has been shown to reduce hydrogen bonding, increase lipophilicity and reduce the hydrodynamic radius in solution, thereby enhancing

membrane permeability (74-77). The cyclic analogues of Pen-enkephalin were shown to resist enzymatic degradation and were capable of crossing the blood-brain barrier (BBB) efficiently and rapidly distributed throughout the body (78).

Several chemical strategies have been developed to convert a linear peptide to a cyclic structure (79, 80). One of the best examples to illustrate these aspects is probably again the Arg-Gly-Asp (RGD) tri-peptide. Formation of disulphide bridges between two cysteine residues flanking the RGD domain leads to increased receptor binding affinity and selectivity (7, 81). Enzymatic stability of herpes simplex virus enhanced impressively when cyclized by glycoprotein D, 29% of the peptide remained intact in the cyclic variant with disulfide linkage whereas 73% of intact peptide found in the variant with peptide bond (82). Cyclosporin A, a natural prototype of orally active peptides, is cyclic and not less than seven out of its eleven peptide bonds are N-methylated, moreover, it is substituted by one non-coded amino acid (4-butenyl-4-methyl-threonine) (83). Peptides with sulfide bridges like Neurotoxin peptides (e.g. the conotoxins), somatostatin, oxytocin, LHRH and insulin were shown to be very promising peptides (84, 85). An orally active peptide was developed by backbone cyclization of natural conotoxin, which was used for the treatment of neuropathic pain (86). Cyclotides, cyclic plant peptides having three conserved disulfide-bonds, were found to be active against HIV. Their activity was independent of a protein receptor and dependent on peptide oligomerization at the membrane surface (87).

1.4.4 PEGylation

Proteolytic stability of peptides can also be increased by covalent conjugation of polymers like PEG or poly (ethylene glycol). PEG chains entangle around the peptide via hydrophobic interactions and forms hydrophilic interactions with the surrounding water which increase the apparent size and solubility of the peptide thereby reducing renal ultrafiltration and biodistribution (88-90). It is now well established that a minimum of 20 kDa PEG is needed for the significant retardation of renal clearance via glomerular filtration (91). PEG conjugation also shields the antigenic epitopes of the polypeptide reducing immunogenicity and degradation by proteolytic enzymes, therefore, pegylation plays an important role in drug delivery (92). The main advantages of Pegylation are depicted in (**Figure 1.4**)

Though pegylation improves potency of many peptides, it is also accompanied by loss of biological activity with improper choice of PEG (i.e. number of PEG chains), unfavorable choice of attachment site, the molecular weight and structure of PEG chains and the chemistry used to attach the PEG to the polypeptide. A good example is PEGylated interferon α-2a, an antiviral cytokine for the treatment of hepatitis C and B that increases antigen presentation of the host and activates immune cells (Pegasys, Hoffmann-La Roche, Inc.).

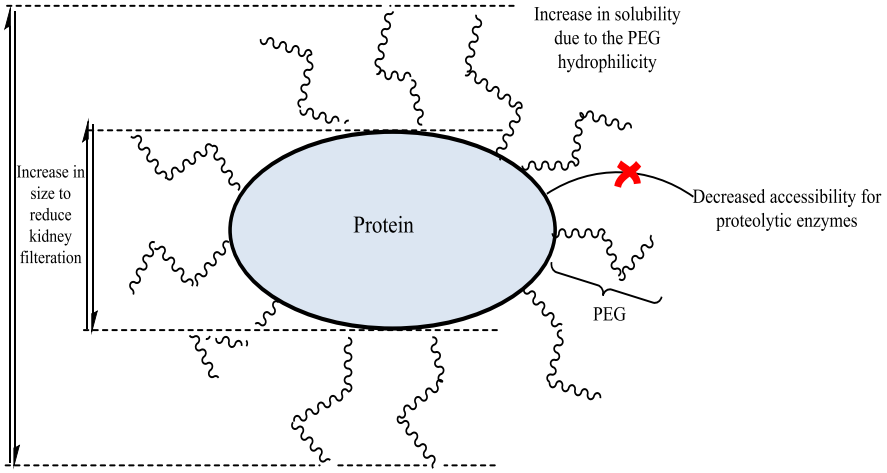


Figure 1.4: Main advantages of PEGylated peptide. The figure represents a PEG-peptide conjugate. The polymer, PEG, is shielding the peptide surface from degrading agents by steric hindrance. Moreover, the increased size of the conjugate is at the basis of the decreased kidney clearance of the PEGylated peptide.

1.4.5 Isosteric manipulations of the native amino acid backbone

These molecules are generated due to modifications in the amino acid backbone of the native structure for improved bioavailability, selectivity and stability. They have a modular structure with amino acids or their derivatives as building blocks involving alteration or replacement of some of the atoms participating in the peptide backbone. Considering a delicate balance between rigidity and flexibility these are designed rationally by studying the detailed structure and knowing the positions of the key chemical groups of the original peptide. The various approaches include:

(i) Changing the amino functionality: Replacement of at least one amino group in the peptide chain by an oxygen atom or sulphur atom. Boger et al. reported that

thiocoraline and its analog, BE-22179, have high binding affinity for DNA and show notable cytotoxic activity against the L1210 cell line at pico-molar levels (93).

(ii) Replacement of α-CH: Replacement of Cα of an amino acid by NH gives azapeptides. Replacing all α carbons with nitrogen atoms gives aztides. For example, Atazanavir (ReyataztTM), an FDA-approved antiretroviral drug is a highly active azapeptide inhibitor of the HIV protease.

(iii) Extension of the backbone by one or two atoms: Extension of peptide backbone by inserting extra carbon or other atoms between the carboxyl and the amino groups of an amino acid offers outstanding stability toward proteolytic enzymes (94-97). α-Aminoxypeptides (β-carbon is replaced by an oxygen atom), β- and γ-aminoxypeptides (addition of an extra carbon atom to the aminoxy backbone), α-Hydrazinopeptides and aza-β3-peptides are generated by amino acid backbone extension. Some of these modified peptides have found significant clinical use as FDA-approved drugs, such as romidepsin (Istodax™), atazanavir (Reyataz™) and bortezomib (Velcade™) (98).

Thus, chemical modifications circumvent the limitations of traditional peptides used in cancer therapy and enhanced the potential of modified peptides in the design of pharmaceutical agents.

1.5 **Different molecular markers for tumor targeting**

1.5.1 Integrins

Integrins are cell surface markers, expressed in all cell types except erythrocytes. They consist of 24 distinct combinations of α and β heterodimeric subunits of which 18 α and eight β subunits are known in humans. These different combinations contain potential sites for the ligand and are responsible for different ligand specificity. Approximately one third of these distinct combinations recognize the RGD sequence to a varying extent (99). The cytoplasmic tails of α and β subunits remain associated to maintain the integrin in inactive state. But this can be disrupted by agonist such as chemokines, which can activate the integrins. The main functions of integrins include cell surface attachment to extracellular matrix (ECM) and inside-out cell signaling. However they are also involved in many other functions such as cell adhesion, migration, survival and apoptosis. The α-integrin–β-integrin heterodimer conformation is affected by the intracellular adaptors as well as the extracellular factors.

Some of the integrins that are highly upregulated in some tumors are $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and $\alpha_5\beta_1$. They have different roles in all stages of cancer. Integrin $\alpha_6\beta_4$ and $\alpha_v\beta_3$ are highly expressed in primary breast cancer cells and are responsible for increased tumor size (100). The $\alpha_6\beta_4$ integrin or its downstream signaling effectors are important therapeutic targets for breast cancer targeted therapies. The β_4 subunit is expressed only in combination with α_6, so detection of the β_4 subunit indicates expression of the $\alpha_6\beta_4$ integrin (101). Integrins act as cell

adhesion receptors and binds to extracellular ligands such as fibronectin, vitronectin, plasminogen, prothrombin, laminin, and osteopontin and various growth factors, immunoglobulins and cytokines. This receptor-ligand binding results in down-stream cell signalling pathways (102, 103). It was reported that integrins bind to extra cellular matrix proteins through the RGD peptide sequence (104). Therefore RGD motif in a peptide can be efficiently utilized to block integrin and native ligand interaction and can be used in tumor targeted therapy and diagnostics (105-107).

1.5.2 Aminopeptidase N

Aminopeptidase N (APN or CD13) is a multifunctional membrane bound protein with enzymatic as well as other functions, including antigen presentation, protein degradation, cell proliferation, cell migration, and angiogenesis (108-110). It has been associated with the growth of different human cancers and suggested as a suitable target for anti-cancer therapy. It also serves as a receptor for tumor homing peptides. Different approaches have been used to develop new drugs directed to this target, including enzyme inhibitors as well as APN-targeted carrier constructs. Studies have shown that tripeptide NGR (Asn-Gly-Arg) can specifically bind to cells expressing APN and therefore NGR is gaining popularity as an efficient ligand for developing peptide based delivery system targeting tumors (111).

1.5.3 Human epithelial growth factor

The EGF receptor is a transmembrane protein tyrosine consisting of three domains, an extracellular N-terminal binding domain, a hydrophobic transmembrane region and an intracellular C-terminal tyrosine kinase (TK) domain. EGFR and its three related proteins, namely ErbB-2, ErbB-3, and ErbB-4 play important role in both normal physiological and cancerous conditions. The epidermal growth factor receptor type 2 (ErbB-2) is a carcinoma associated receptor which binds to EGF and causes activation of the kinase and subsequently receptor autophosphorylation which leads to tumor cell survival, proliferation, invasion and metastases in many human cancers (112, 113). Peptides which bind and block the signaling pathway of ErbB are important for cancer therapy. EGFR is expressed in a variety of human tumors, including those in the breast, colon, lung, head and neck. Two different types of EGFR blockers have been developed: mAbs, such as cetuximab and panitumumab which targets the extracellular N-terminal domain of the receptor thereby inhibiting ligand dependent EGFR signal transduction and Tyrosine kinase inhibitors, such as gefitinib and erlotinib, which target the intracellular tyrosine kinase domain of the EGFR. In addition to this inhibition therapies, small peptides such as YHWYGYTPQNVI (GE11) and LARLLT have been utilized as effective ligands for tumor targeting therapy (114, 115).

1.6 **Cancer Targeting Peptides Based on P160 dodecapeptide**

Numerous strategies have been employed for selective delivery of chemotherapeutic cargo to cancer cells in tumors. Most effective strategies involve specific targeting of anticancer drugs using small ligands such as tumor targeting peptides. In this regard, a number of tumor homing peptides have been reported showing promising outcome for targeted delivery of drugs to tumors. To further explore the feasibility of peptides for targeting tumors cells a number of peptides have been identified by peptide phage display for targeting breast cancer cells (116, 117) .

To achieve cancer cell targeting a dodecapeptide p160 (VPWMEPAYQRFL) was identified by random peptide phage display by Zhang et al. in 2001 (117). Specifically, the peptide P160 consisting of 12 amino acids was isolated via selection rounds of a phage library on the human neuroblastoma cell line WAC 2. Authors demonstrated high affinity for breast cancer cell lines MDA-MB-435 and neuroblastoma cell line WAC 2 and low binding to primary HUVEC cells. In another study it was shown that when intravenously injected, ^{131}I-labeled p160 preferentially accumulated in tumors than in normal organs like heart, liver, spleen, lung, kidney, muscle and brain (118). The stability studies of p160 in human serum revealed complete degradation by serum proteases after 4 h.

In order to further improve peptide p160, our lab developed a novel method, peptide array whole cell binding assay to screen for better analogues of p160 for specific recognition by cancer cells. A library of 70 peptide sequences has been

synthesized on cellulose membrane and screened against two human breast cancer cell lines, MDA-MB-435 and MCF-7 (119). From above sequences, five new analogues of peptide p160 were identified and these displayed high affinity for MDA-MB-435 and MCF-7. Amongst the five, two peptides (p11) RGDPAYQGRFL and (p18) WXEAAYQRFL showed better binding profile compared to the wild type p160 while showing low binding for HUVEC cells. However, p18 was shown to be less stable in human serum and completely degraded within 30 min, giving two main degradation products that appear after 10 min of incubation with serum (120). The degradation products of peptide 18 show masses of 997.1 and 878.1 g/mol, which correspond to EAAYQRFL and WXEAAYQ sequences, respectively.

Therefore, to improve the proteolytic stability and maintain the specific affinity for breast cancer cells second generation analogues of p160 have been designed by replacing one or two amino acids in the peptide p18 and also proteolytic stable analogues of p18 have been developed by introduction of α and β^3 amino acids in the sequence. This study has demonstrated design of three proteolytic stable peptide analogues, 18-4 (WxEAAYQrFL), 18-9 (ZXEAAYQKFL) and 18-10 (ZXEAAYQKFL) where replacement of 2 (18-4) or 3 residues (18-9 and 18-10) in a 10-mer peptide with D- or β3-amino acids led to complete proteolytic stability. Peptide 18-4 with two D-amino acid substitutions at the enzymatic labile sites was stable in the presence of human serum and liver homogenate from mice, and showed up to 3.5-fold enhanced binding to cancer cells. In addition, 18-4 is potentially safe with minimal cellular toxicity (120). Using 18-4 as lead sequence,

a dodecapeptide 18-4a (WxEAAYQkFL) was designed where D-Arg substituted with D-Lys to facilitate side chain attachment to Dox which creates a breast cancer prodrug to selectively target and deliver Dox to breast cancerous cells with reduced delivery to normal cells. Recently, we reported that 18-4a-Dox conjugate through an ester linkage enhanced the Dox selectivity 40 times to breast cancerous cells MDA-MB-435 than the normal cell line MCF-10A compared to free drug (121).

1.7 **Thesis Proposal**

1.7.1 Thesis Rationale

The survey of contemporary literature suggests that main challenge for designing successful cancer drug therapy is to selectively target drugs to tumor tissues. The non-specific distribution of drugs in healthy organs or tissues leads to severe side effects, which results in failure of the treatment and might end up increasing drug resistance. Such undesirable side effects often limit the concentration of a drug that can be administered. Therefore, targeting drugs to the tumor can help to improve the outcome of existing cancer therapies. To accomplish this, different strategies are being investigated in order to achieve selective delivery of the drug to tumor. The introduction of various tumor targeting biological ligands like antibodies, aptamers, affibodies and peptides into drug delivery systems has provided the opportunity for the selective delivery of drugs to tumor sites.

Peptides are a class of small ligands that hold great potential for tumor targeting and can serve as alternate strategy to conventional chemotherapy (122). Conjugation of cancer drugs to tumor specific peptides has been shown to improve their therapeutic efficiency. In recent years, phage display technique has been used for the identification of number of peptide sequences for recognizing and targeting tumor- associated proteins. Therapeutic peptides offer several advantages over small entities that make up the most traditional medications, therefore, the growth rate in the peptide market is significantly higher. The attractive properties that make peptides very appealing candidates for developing

new target-specific therapies are their small size, relatively easy and rapid synthesis process, low risk of systemic toxicity, greater efficacy, selectivity and specificity and possibility for chemical modification for designing novel peptide analogs (123). However, the drawbacks related to the use of peptides are metabolic instability and short half-lives in systemic circulation. The limitations associated with peptides can be improved by modifying the peptide backbone sequence using amino acids with D configurations, cycling of the N-terminal with the C-terminal or with a side-chain, the C-terminal with a side-chain and the side-chain with another side-chain.

1.7.2 Hypotheses

We hypothesise that the lead decapeptide 18-4DXK identified earlier can be further improved for cancer cell specific binding and proteolytical stability by optimizing its chemical structure to obtain various engineered analogs. We anticipate that these engineered analogs obtained by L amino acid substitution at any of the labile sites or N- to C-terminal cyclization will improve overall performance of lead peptide sequence.

1.7.3 Thesis Objectives

The objective of this study was to engineer cancer targeting peptides based on lead sequences 18-4DXK for enhanced proteolytic stability and binding affinity for breast cancer cells (**Table 1.2**). Our lead peptide was obtained by engineering the peptide p160. In the current study, five peptide analogues were synthesized based on lead decapeptide 18-4a (renamed in the current study as 18-4DXK) to enhance the affinity and specificity towards breast cancer cell lines while maintaining the proteolytic stability. The design strategy involved replacement of either nor leucine (x) or lysine (k) amino acid in the lead sequence with the corresponding L residues or cyclization of the sequence. **Table 1.2** shows the peptide analogs and their amino acid composition.

Table 1.2: Summary of the design and synthesis of four analogues based on lead sequence.

Peptide	Amino acid sequence	Comments
18-4DXK	WxEAAYQkFL	Lead sequence with two D amino acids
18-4DX	WxEAAYQKFL	One D amino acid at the labile site
18-4DK	WXEAAYQkFL	One D amino acid at the labile site
c18-4DK	cWXEAAYQkFL	Cyclization
c18L	cWXEAAYQKFL	Cyclization of the alpha sequence
18L	WXEAAYQKFL	Control for proteolytic stability study

*The red letter denotes D amino acids

The specific objectives are as follows:

- To synthesize new peptide analogues based on the lead decapeptide 18-4DXK sequence.
- To screen the peptide analogues for binding using cancerous and non-cancerous cells. Specifically, study the peptide uptake using the breast cancer cell lines (MDA-MB-231, MDA-MB-435, and MCF-7) and with non-cancerous cells (HUVEC and MCF-10A).
- To compare binding affinity of different peptide sequences namely p160, p18, 18-4DXK to that of new peptide analogues c18-4DK and c18-L.
- To study the effect of trypsin on magnitude of cell uptake. Specifically compare effect of trypsinization versus mechanical cell scrapping on uptake of peptides.
- To evaluate the peptide analogs for proteolytic stability in the presence of human serum.
- To study mechanism of the uptake of peptides using breast cancer cells.

Chapter 2 Experimental Methods

2.1 Material and Equipment

Rink amide resin (0.79mmol/g), O-(1H-6-Chlorobenzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HCTU) and the Fmoc-amino acids were purchased from NovaBiochem (San Diego, CA, USA). Dichloromethane (DCM), methanol, isopropyl alcohol (IPA), acetonitrile (ACN), diethyl ether and piperidine, N, N,- dimethyl formamide(DMF), N-methyl morpholine (NMM), tetrahydrofuran (THF), trifluoroacetic acid (TFA) . All other chemicals were commercially available by Sigma-Aldrich and were used as received, unless otherwise stated. 5-FITC (Fluorescein-5-isothiocyanate) was from Ana spec (Fremont, CA). The absence of amines in DMF and NMP was detected using Bromophenol blue (BPB).The peptide synthesis was performed manually in small plastic columns with a frit at their base for solvent removal under suction and a cap with septum at the top for the addition of reagents. HPLC purification and analysis were carried out on a Varian Prostar HPLC system using Vydac C18 semi-preparative (1 x 25 cm, 5 μm) column. Peptides were detected by UV absorption at 214 nm. Mass spectra were recorded on a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) Voyager spectrometer Applied Bio systems). FACS experiments were performed on FACS Canto II and LSR Fortessa flow cytometer (BD Biosciences) and data was analyzed using FlowJo software (Tree Star Inc).

2.2 **Peptide Synthesis**

2.2.1 Linear Peptide Synthesis

Solid phase synthesis of peptides was done manually on Rink amide resin. Four 10-mer linear peptides (18-4DXK, 18-4DX, and 18-4DK and 18L) and their fluorescent analogues were synthesized by solid-phase peptide synthesis (SPPS) using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry and subsequently purified by RP-HPLC.

The Rink amide resin (substitution 0.79mmole/g; scale 0.2mmol, 253mg) was used as polymeric support. In a plastic column with a frit, resin is weighed. It is then washed with DMF, DCM and IPA and swelled for 1 h in DCM. Next, the first amino acid, N- α-Fmoc-Leu (3 eq.), was activated in O-(1H-6-Chlorobenzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HCTU, 3 eq.) in DMF and N-methyl morpholine (NMM, 3 eq.) for 5 min and then added to the swelled resin. The reaction was left for 2-3 hr. The Fmoc protecting group of the first amino acid was removed with a solution of 20 % piperidine in DMF and the peptide was assembled using standard Fmoc procedures by sequential addition of Fmoc protected amino acids with three fold excess of O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) in DMF and N-methyl morpholine (NMM) as coupling agents for 2 h. After each coupling, the Fmoc protecting group is removed from the N terminus using 20% piperidine in DMF (2 x 7min). Washing between deprotection and coupling was carried out with DMF (3 x 1min), DCM

(3 x 1min) and IPA (3 X 1min). Each amino acid coupling was performed for two hours at room temperature. Ninhydrin or Kaiser Test was performed to estimate the completeness of the reaction. After addition of the last amino acid, the N-terminal Fmoc group was then removed with 20% piperidine in DMF (2 X 10 min). Fmoc-β-alanine linker (3 eq) is linked to the free amino group of the last amino acid (Tryptophan) in the peptide chain using HCTU (3 eq) / NMM (3eq) for 2hrs. Colorimetric Kaiser Test was done before and after β-alanine coupling for the presence of free amino groups, followed by FITC coupling. The β-alanine residue was introduced before the coupling step of FITC chromophore. The peptide resin was then divided into two reaction columns, each containing 0.1mmol of peptide resin. One of the reaction column is used to obtain fluorescent analogue of the synthesised peptide by coupling FITC and the other reaction column is used to get unlabelled peptide.

For FITC labeling 0.1mmole of resin was used. After Fmoc deprotection of β-alanine moiety of the peptide sequence using 20% of piperidine, the resin was washed with DMF (3X). Meanwhile, highly concentrated slurry of FITC (0.3mM, 116.8mg) and DIPEA (0.15 mM, 19µl) in anhydrous DMF (1 mL) were added to the peptide resin, followed by stirring for 48 hrs at room temperature in the dark (covering the RV with aluminum foil). After completion of the reaction, FITC labelled peptide was washed thrice with DMF and IPA and dried completely. The peptides were fully deprotected and cleaved from the resin with TFA-TIPS-H2O (95:2.5:2.5, 7.5 mL) mixture at room temperature for 2 h and washed with TFA: CH2Cl2 (1:9, 7 mL). The acid washings were concentrated to approximately 1

mL and precipitated by ice cold diethyl ether. The precipitate was collected by centrifugation and again washed with ice-cold dry diethyl ether (4× 30 mL). The precipitate was isolated and dried in a stream of nitrogen and lyophilized. The purity of the final product was assessed by reversed-phase high performance liquid chromatography (RP-HPLC) on a using Vydac C18 semi-preparative (1 x 25 cm, 5 μm) column and eluted with H2O/0.1% TFA (A) and ACN (B) from 15 to 55% over 35min at a flow rate of 2ml/min. The mass of the products was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The final yields of purified peptides ranged between 60 and 70%. The side chains of the amino acids used in the synthesis of linear peptides were protected as follows: tert-butyl (tBu) for tyrosine, tert-butoxy (OtBu) for glutamic acid, trityl (Trt) for glutamine, t-butoxycarbonyl (Boc) for tryptophan and lysine.

2.2.2 Cyclic Peptide Synthesis

Two cyclic peptides c18-4DK and c18L and their fluorescent analogues were synthesized by solid-phase peptide synthesis (SPPS) using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry and subsequently purified by RP-HPLC. Briefly, a solution of Fmoc-Glu-OAll (122.8 mg, 3 equiv) and HCTU (3eq.) in DMF and NMM (3 eq.) were added to pre-swelled Rink amide resin (substitution 0.79mmole/g; scale 0.2 mmol, 253mg) and the reaction was left for 2h. The Fmoc protecting group of the coupled amino acid was removed using 20% piperidine in DMF and the presence of free amino group is confirmed by Kaiser Test, then the next amino acid is then coupled using 3 eq. of coupling agents. All coupling

reactions were activated using HCTU (3 eq.) in DMF and NMM. Washings of DMF (3 x 1min), DCM (3 x 1min) and IPA (3 X 1min) was given between deprotection and coupling. Likewise, the peptide was assembled sequentially using standard Fmoc procedure. After addition of the last amino acid, deallylation was done with Pd (18.4mg, 0.16 equiv) and PhSiH3 (173µl, 16equiv) in DCM for 2hrs in a gas tight syringe with septum. The reaction mixture was washed with diethyldithiocarbamic acid sodium salt, 10mg in 2000µl DCM (0.5%w/v in DMF). Kaiser test for any free amino group was negative at this stage.

The N- terminal Fmoc group was then removed with 20% piperidine in DMF, followed by on resin cyclization using BOP (1.95equiv, 86mg), HOBt (2 equiv, 27mg), and NMM (4.5equiv, 50µl) in DMF for 2hrs to give cyclized peptide. After coupling, the resin was washed with DMF and DCM. Coupling was monitored with the Kaiser test. Removal of (Dde) protecting group from the amino side chain of lysine was achieved by hydrazine monohydrate in DMF (2:98) for 3 minutes. Fmoc-β-alanine linker (3equiv, 93mg) is linked to the free NH_2 of the lysine side chain using HCTU (124mg, 3 equiv) / NMM (30µl, 3equiv) mixture as coupling agents for 2hrs. Kaiser test was done before and after β-alanine coupling for the presence of free amino groups, followed by FITC coupling.

2.2.3 Fluorescein-5-isothiocyanate (FITC) Labeling of Peptides

Fluorescent label is attached to the peptide so that the peptide could be observed in vitro experiments by flow cytometry. The isothiocyanate group (NCS) on FITC reacts with the free amino group of β-alanine linker.

For FITC labeling 0.1mmole of resin was used. After Fmoc deprotection of β-alanine moiety of the peptide sequence using 20% of piperidine, the resin was washed with DMF (3X). Meanwhile, highly concentrated slurry of FITC (0.3mM, 116.8mg) and DIPEA (0.15 mM, 19µl) in anhydrous DMF (1 mL) were added to the peptide resin, followed by stirring for 48 hrs at room temperature in the dark (covering the RV with aluminum foil). After completion of the reaction, FITC labelled peptide was washed thrice with DMF and IPA and dried completely.

After complete synthesis of FITC labelled peptide, the crude product was cleaved using TFA/ TIPS/ water (90:5:5) for 2hr. The precipitate was collected by centrifugation and washed with ice-cold dry diethyl ether to give orange powder. It was then isolated and dried. The mass of the crude product was identified by MALDI-TOF mass spectrometry. The side chains of the amino acids used in the synthesis of cyclic peptides were protected as follows: tert-butyl (tBu) for tyrosine, tert-butoxy (OtBu) for glutamic acid, trityl (Trt) for glutamine, t-butoxycarbonyl (Boc) for tryptophan and lysine. The chemical structures of the peptides containing D and L amino acids are shown in **Figure 2.1.**

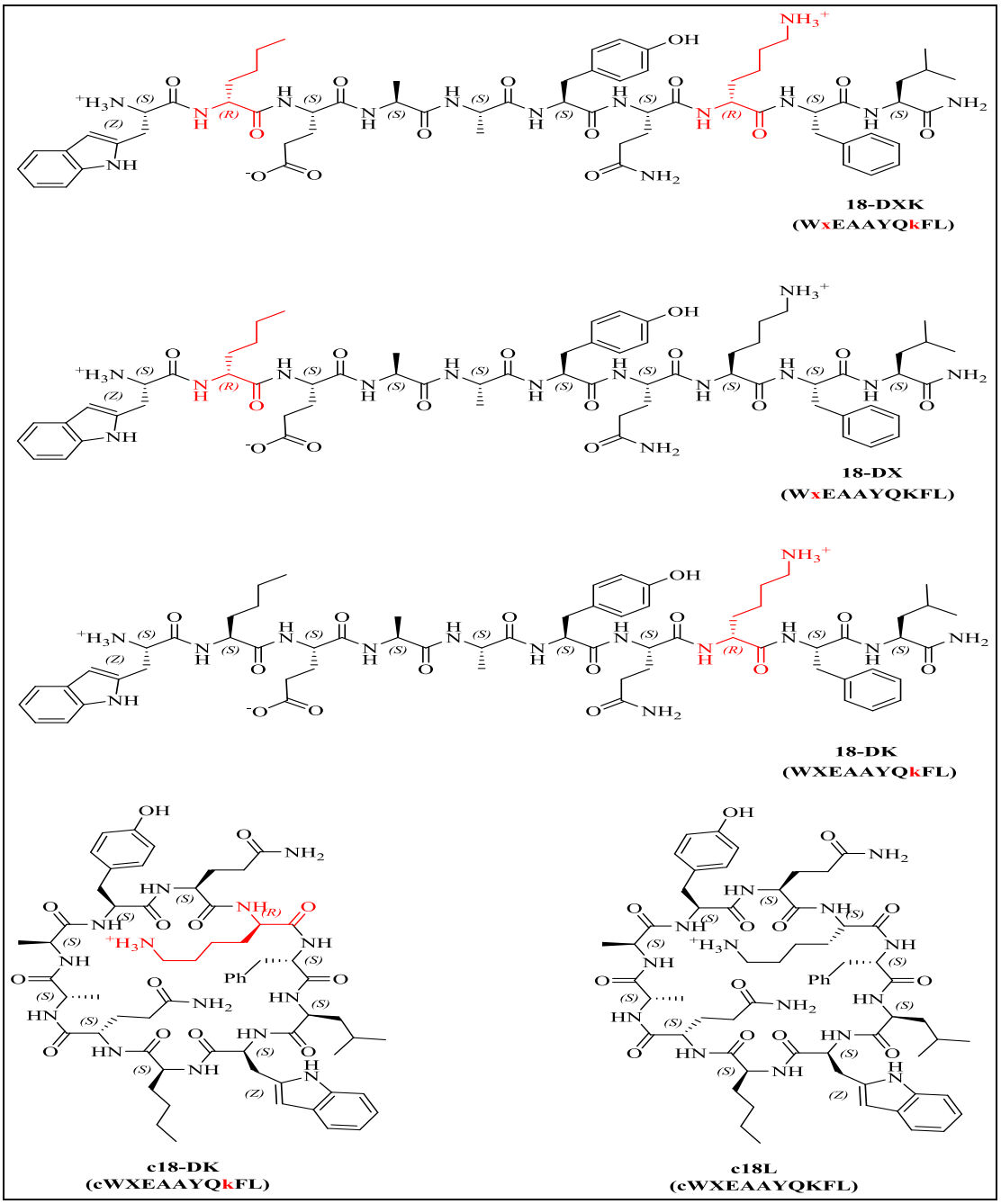


Figure 2.1: Structures of 18-4 peptide analogues namely 18-4DXK, 18-4DX, 18-4DK, c18-4DK and c18L. Amino acids shown in red are D-amino acids. Lower case letters denote D-amino acids, X is Nle (Norleucine).

2.3 **Cell Lines and Culture**

All cancer cell lines and human mammary epithelial cell line MCF-10A were purchased from the American Type culture collection (ATCC). Cells were cultured in a humidified incubator at 37°C in 5% CO_2. All culture media for cancer cells were purchased from Gibco (Grand Island, NY, USA). DMEM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 IU/ml streptomycin was used to culture MCF-7 and MDA-231 cancer cell line; whereas, MDA-MB-435 was cultured in RPMI-1640 media supplemented with 10% FBS, 100 IU/ml penicillin, and 100 IU/ml streptomycin. Trypsin EDTA (1X, 0.25% trypsin/ 0.53% EDTA in HBSS), Penicillin-streptomycin solution was purchased from Life technologies.

The non-cancerous human epithelial cell line MCF 10A was cultured in Mammary Epithelial Cell Medium (MEGM kit from Lonza, USA) supplemented with cytokines, Bovine Brain Extract (BPE), Hydrocortisone, human Epidermal Growth Factor (hEGF), Insulin, Fetal Bovine Serum (FBS), and Gentamicin/Amphotericin-B. Human umbilical vein endothelial cells (HUVEC), a kind gift from the laboratory of Sandra Davidge, University of Alberta, were cultivated in Endothelial Cell Growth Media (EGM kit from Lonza, USA) containing 20% FCS, 2 mmol/L glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 2 ng/mL basic fibroblast growth factor (Roche Diagnostics, Mannheim, Germany).

2.3.1 In-vitro cell uptake assay for screening peptides

Fluorescence-activated cell-sorting (FACS) analysis was used to evaluate the uptake of FITC-labeled peptides against three human breast cancer cell lines (MDA-MB-435, MCF-7, and MDA- MB-231) and two noncancerous cell lines (MCF-10A and HUVEC) using LSR-Fortessa (BD Biosciences) and data was analyzed Flowjo software.

All the cell lines were grown in 75 cm^2 culture flasks containing medium supplemented with 10% FBS and 1 % penicillin-streptomycin solution until 80% confluence at 37°C. The media was then aspirated and cells were washed with PBS (pH7.4). The cells were dislodged using 0.25% of trypsin EDTA solution and if needed kept at 37°C for few minutes to trypsinize them completely. The trypsin was then deactivated using small volume of FBS containing complete growth medium. The suspended cells were collected in a falcon tube and centrifuged for 500g for 5 min. The resultant pellet was re-suspended in media and the cells were counted using haemocytometer. Based on the number of cells appropriate volume of media was added and cells were seeded in a 12-well tissue culture plate at a density of $1x10^6$ in 1 mL of culture medium at 37 °C for 24 h in a humidified CO_2 incubator. After 24 h, the media was aspirated and the cells were washed twice with PBS. The cells were then incubated in serum free medium containing FITC labeled peptides at 1 μM concentration for 30 min at 37 °C in incubator. After 30 minutes of incubation, the cells were thoroughly washed three times with PBS to remove unbound peptide. The cells were then detached

form the surface using trypsin EDTA and trypsin was deactivated using FBS containing complete growth media. The cells were then collected in FACS tube and centrifuged at 500g for 5min. The pellets were re-suspended in FACS solution (2% FBS in PBS), and centrifuged again at 500g for 5 min, the washing was repeated again to remove any unbounded peptide. The cells were finally re-suspended in FACS solution (2% FBS in PBS) and analysed using BD LSR-Fortessa flow cytometry. The unlabeled cells were used as control and their auto fluorescence represents the cut-off point value and thus differentiates them from the FITC labeled cells. The experiment was performed in triplicates and 10,000 events were recorded for each sample.

Separately, the cell binding studies were carried out at 4°C. For this cells were placed on ice and incubated with respective peptide for 30 min and rest of the downstream steps were carried as described above.

2.4 **Proteolytic stability**

The selected peptides were accessed for the proteolytic stability in the presence of human serum. Human serum was centrifuged at 1700 g for 10 min for the removal of the lipid component. In an Eppendorf tube, 650 µL RPMI and 250µL of human serum was taken and to it 100 µL of an aqueous peptide stock solution was added. The tube is then kept in a water bath at 37°C. The initial time was recorded and at regular time intervals (0, 2h, 4h, 6h, 24h) an aliquot (100µL) from the reaction tube was taken out and added in a separate Eppendorf tube containing methanol (200µl). The proteins in the serum will precipitate out in the presence of

methanol and gives a cloudy appearance to the solution. The cloudy solution produced was then cooled to 4°C for 15 mins and then spun at 500g for 15 min to pellet the precipitated serum proteins. The resulting supernatant is then injected into a RP-HPLC Vydac C18 column with a linear gradient from 15-55% ACN/ water (0.1% TFA) in 35 min with a flow rate of 2ml/min and the absorbance of the eluting peaks was detected at 214 nm. The concentration of peptides and degradation products were measured by integrating the area under the curve and their identity was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

2.5 **Comparative uptake with trypsinization and scrapping**

The purpose of this experiment was to analyze whether cells are indeed taken up by the receptor-mediated process and whether the trypsinization of cells following uptake can influence the overall magnitude of uptake. Therefore for comparison the experiment was performed as described in section 2.3.1 above for trypsinization. Parallel to this a set of cells were treated with PBS solutions containing 2 mM EDTA and cells were incubated on ice for 5 min and then gently scrapped and clumps were broken down by pipetting. The remaining steps were performed as described in the previous section. The data were analyzed using FlowJo software for any difference between trypsin treated cells and scrapped cells. The differences in the mean fluorescence intensity values were analyzed using Graph Pad prism software for statistical significance.

2.6 **Competitive uptake study**

The main goal behind this experiment was to study whether presence of free unlabelled peptide can reduce the extent of uptake of FITC-conjugated peptide. The ultimate outcome of this experiment will prove that uptake of peptide is indeed mediated by cell-associated receptors. Thus, in case the peptide uptake is receptor-mediated the presence of free peptide should reduce the overall magnitude of uptake.

Briefly the cells were processed and seeded as described in detail in previous section. To study the competitive uptake the breast cancer cell lines MDA-MB-231 and MCF-7 were pre-incubated for 15 min in the presence of 50 fold excess of unlabeled c18-4DK (50 μM) and then the FITC-labelled c18-4DK peptide was incubated with cells at 1 μM for 30 min. After 30 min cells were washed to remove free peptide and trypsinized for dislodging. Cells were washed with FACS buffer and fluorescence was measured using LSR-Fortessa flow cytometer. Data was analyzed using FlowJo software. The statistical tests were performed using Graph Pad prism software for difference in the mean of percent FITC positive cells.

Chapter 3 Results and Discussion

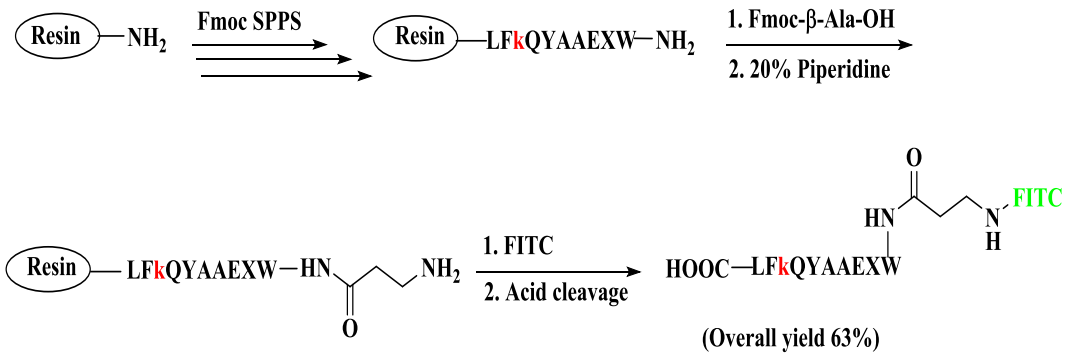
3.1 **Design and synthesis of 18-4DXK analogues**

In our earlier experiments it was reported that Dox-peptide 18-4DXK (WxEAAYQkFL) conjugates specifically target tumors and demonstrate enhanced cytotoxicity against drug resistant cells compared to free doxorubicin. In this study, four analogues of the lead decapeptide 18-4DXK were designed to further enhance binding affinity to breast cancer cells and stability towards proteases. Using the parent peptide 18-4DXK, six analogues have been designed, synthesized and evaluated. Two linear analogues (18-4DX, 18-4DK), two cyclic analogues (c18-4DK, c18-4L) and a control α-peptide (18-4L). The design strategy involved replacement of either norleucine (x) or lysine (k) amino acid in the lead sequence with corresponding L residues and/or cyclization to enhance proteolytic stability as well as increase binding affinity to breast cancer cells. The residues norleucine(x) and lysine (k) were identified as labile sites in the peptide by Askoxylakis and co-workers (117, 118). The synthesis of the peptides was done on solid phase as described (**Schemes 1.1 and 1.2**). The mass spectrometry data and the HPLC elution time of the peptides are listed in **Figure 3.1 and 3.2**

3.1.1 Synthesis of Linear Peptides:

Four linear 10-mer peptides (18-4DXK, 18-4DX, 18-4DK, 18L) were synthesized using Rink amide resin (0.79mmole/g) as polymeric support. The chemical structures of the peptides containing D amino acids are shown in **Figure 2.1**.

Fmoc protected amino acids were activated using HCTU in DMF and NMM and coupled in 3-fold excess. After coupling for 2 h at room temperature, Kaiser Test was performed to estimate the completeness of the reaction. Deprotection was done using 20% piperidine in DMF. Fmoc-β-alanine linker is linked to the free NH_2 of the last amino acid (Tryptophan) using HCTU / NMM for 2hrs (Scheme 1.1). Colorimetric Kaiser Test was done before and after β-alanine coupling for the presence of free amino groups, followed by FITC coupling. Peptides were cleaved off from the resin by TFA/ DCM/ TIPS. The resulting mixture was agitated for 2 h before washing with the cleavage reagent twice. The acid washings were concentrated and precipitated by cold diethyl ether. The precipitate was collected by centrifugation and washed with ice-cold dry diethyl ether to give white powder. The precipitate was air dried first and freeze-dried using lyophilization.

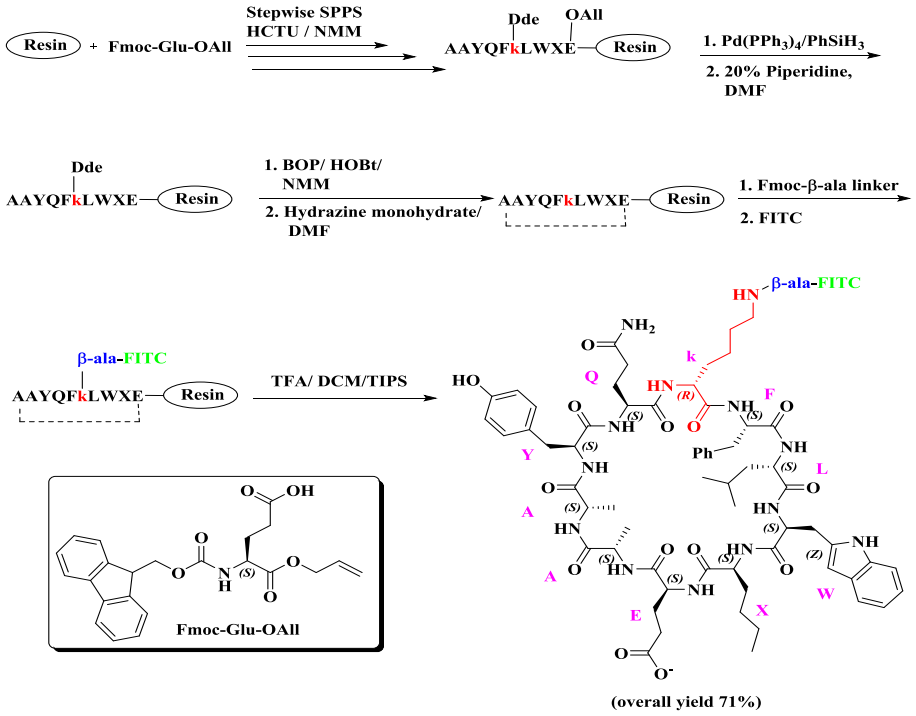


Scheme 1.1: Scheme for solid phase synthesis of FITC labeled linear peptides (18-4DXK, 18-4DX and 18-4DK)

3.1.2 Synthesis of Cyclic Peptide:

Briefly, Fmoc protected amino acid (Fmoc-Glu-OAll) was activated using HCTU in DMF and NMM and coupled in 3-fold excess to Rink amide resin (0.79mmole/g) for 2 h (Scheme 1.2). The Fmoc protecting group was removed and the presence of free amino group is confirmed by Kaiser Test, then the peptide was assembled using standard Fmoc procedures as done in linear peptide synthesis. After addition of the last amino acid, deallylation from first coupled amino acid (Fmoc-Glu-OAll) was done with a mixture of Pd (PPh3)4 and PhSiH3 in DCM for 2 h in presence of nitrogen. The reaction mixture was washed with diethyldithiocarbamic acid sodium salt. Kaiser test for any free amino group was negative at this stage. The N- terminal Fmoc group was then removed with 20% piperidine in DMF, followed by on resin cyclization using BOP, HOBt and NMM in DMF for 2hrs to give cyclized peptide, the resin was then washed with DMF and DCM. Coupling was monitored with the Kaiser test. After the terminal free amino group has been cyclized, Fmoc-β-alanine linker is linked to the side chain of Fmoc-lysine (Dde)-OH. Removal of (Dde) protecting group from the side chain of Fmoc-lysine (Dde)-OH was achieved by hydrazine monohydrate in DMF. Fmoc-β-alanine linker is linked to the free NH_2 of the lysine side chain using HCTU/ NMM mixture as coupling agents for 2 hr. Kaiser test was done before and after β-alanine coupling for the presence of free amino groups, followed by FITC coupling to the free NH_2 of β-alanine moiety.

After complete peptide synthesis, the peptide was cleaved using TFA /DCM / TIPS for 2hrs and precipitated by cold diethyl ether. The precipitate was collected by centrifugation and washed with ice-cold dry diethyl ether to give white powder. The precipitate was isolated and dried. The mass of the crude product was determined by MALDI-TOF mass spectrometry.



Scheme 1.2: Scheme for solid phase synthesis of FITC labeled cyclic peptide c18-4DK.

3.2 **Characterization**

Crude peptides were dissolved in water and acetonitrile and purified using reversed-phase HPLC to obtain pure peptides in 45−71% yield. The purity of the peptides was confirmed by HPLC and MALDI-TOF mass spectrometry (**Figure 3.1 and 3.2**) and found to be greater than 95%. The HPLC analysis and mass spectrometric data of the peptides are summarized in **Table 3.1**. All the peptides were labeled with fluorescein 5-isothiocyanate (5-FITC) through their N-terminus via a β-alanine spacer.

Table 3.1: Amino acid sequences and characterization (Mass Spectrometry and HPLC) of cancer targeting peptides studied herein

Peptide	Amino acid sequence	Mass observed[a]	Yield (%)	Rt (min)[b]
18-4DXK	WxEAAYQkFL	1268.6	60-70	25.9
18-4DX	WxEAAYQKFL	1268.5	65-75	24.1
18-4DK	WXEAAYQkFL	1269.5	63-68	24.1
c18-4DK	cWXEAAYQkFL	1250.5	62-71	28.1
c18L	cWXEAAYQKFL	1250.5	50-60	30.7

[a]Maldi –TOF of peptide sequences without β-alanine and FITC (**Figure 3.2**). [b] RP-HPLC retention time of the same sequence (**Figure 3.1**). Gradient used on a Vydac C18 semi-preparative column was 15-55% ACN/ water (0.1% TFA) in 35 min with a flow rate of 2 ml/min. Substitutions with D-amino acids shown in red with lower case. X is Nle.

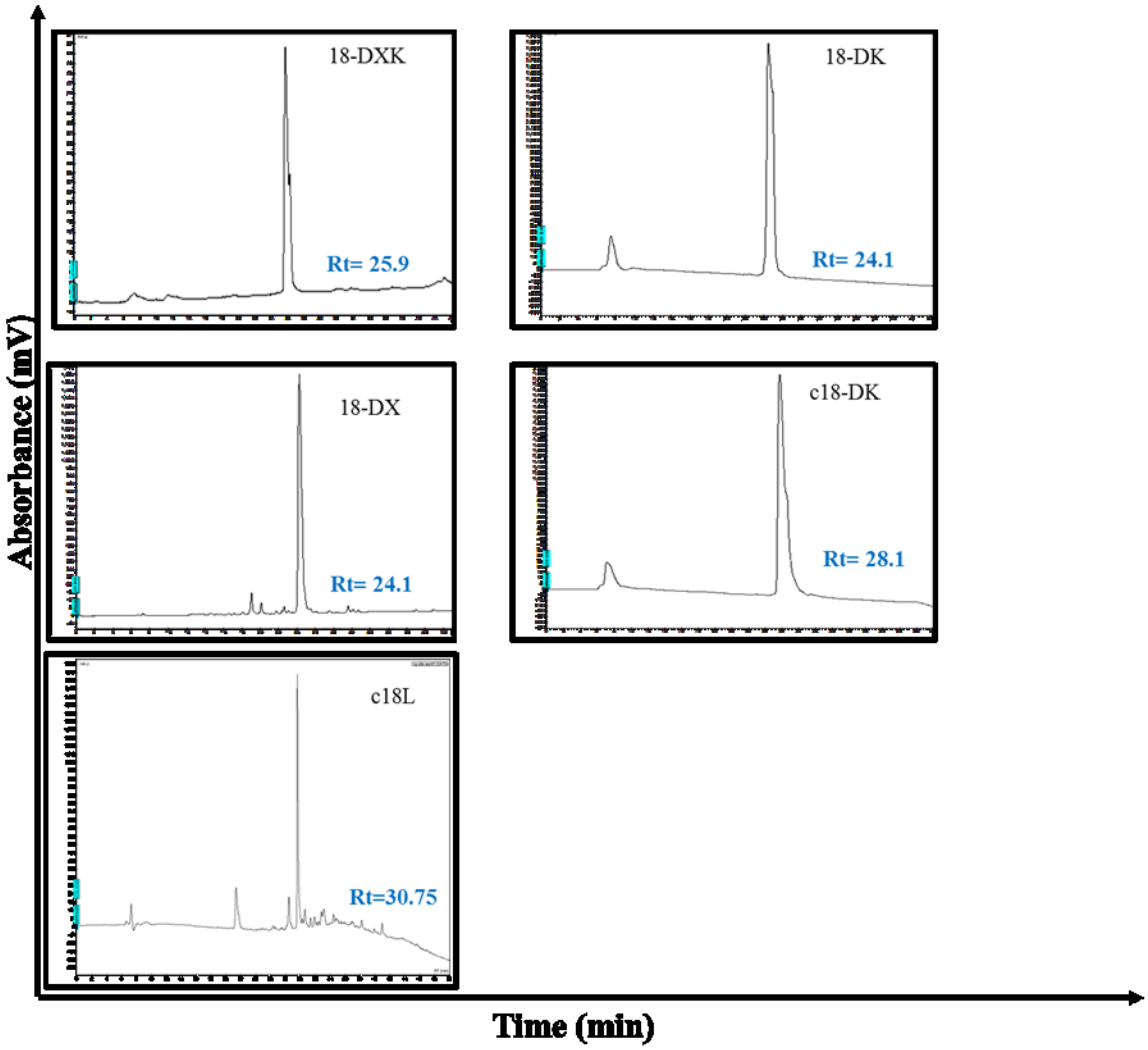


Figure 3.1: RP-HPLC chromatograms of unlabeled peptides 18-DK, 18-DX, 18-DXK and c18-DXK and c18L. The HPLC method used was 15-55% acetonitrile/water in 35 min with a flow rate of 2 mL/min.

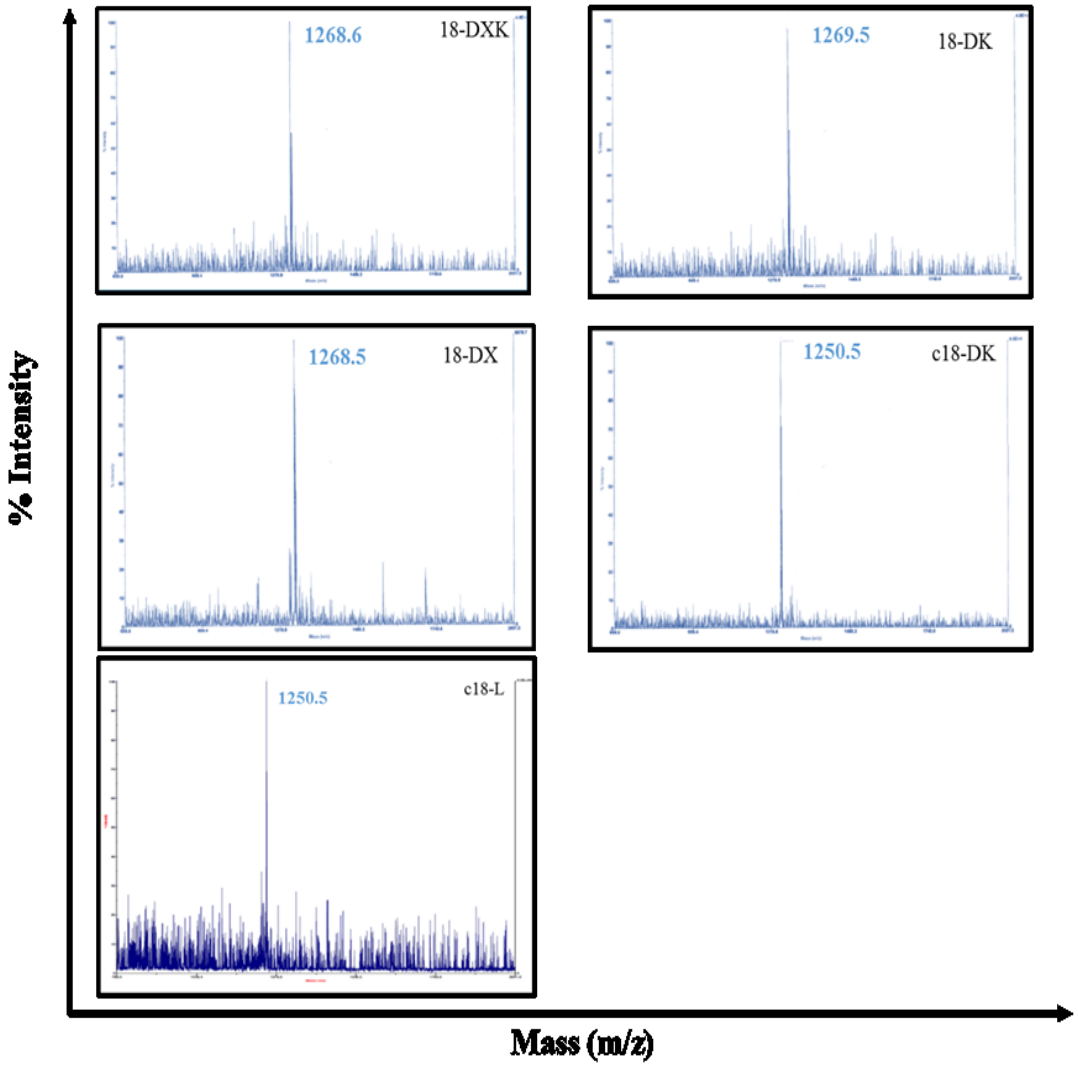


Figure 3.2: MALDI-TOF-MS of unlabeled peptides 18-DXK, 18-DX, 18-DK and c18-DK and c18L showing the [M+H]+ as major peaks

Expected Mass for 18-DXK, 18-DX, 18-DK: 1268.5

Expected Mass for c18-DK and c18L: 1250.5

3.3 **In vitro cell binding assays for screening peptides**

The binding of the new synthesized analogues to different mammalian cell lines was compared with the lead peptide 18-4DXK using flow cytometry. The peptides were labeled with FITC and the tumor targeting ability was studied using breast cancer cell lines (MDA-MB-435, MDA-MB-231 and MCF-7), while non-cancerous cell lines (HUVEC and MCF-10A) were used as control. The results showed that the two analogues 18-4DK and c18-4DK display significant binding to the three breast cancer cell lines as evidenced by the increase in the percentage of fluorescently labeled positive cells relative to the untreated cells (**Figure 3.3A)**. Amongst the two, the cyclic peptide analogue c18-4DK showed highest binding to three breast cancer cell lines (MDA-MB-435, MCF-7 and MDA MB-231) and relatively low binding to normal cell lines (HUVEC and MCF-10A) confirming their selectivity, while the third analogue 18-4DX has shown much less uptake than the parent sequence 18-4DXK (**Figure 3.3B**). It was found that the peptide 18-4DK with substitution of D-Nle2→ L-Nle2, showed a marked increase in binding mainly in two cancer cell lines, MDA-MB-435 and MDA-MB-231 whereas the conversion of this linear sequence into conformationally constrained cyclic sequence resulted in high uptake in all the three breast cancer cell lines. Peptide 18-4DX with substitution D-Lys8→ L-Lys8 substitution has shown a marked decrease in binding with respect to the parent peptide. These results show that D-Lys8 substitution is well tolerated and might be affecting the interaction of peptide with the receptor. Also, cyclization led to a slight increase in

hydrophobicity of the peptide as observed from the increase in the HPLC retention time of the peptide (**Table 3.1**).

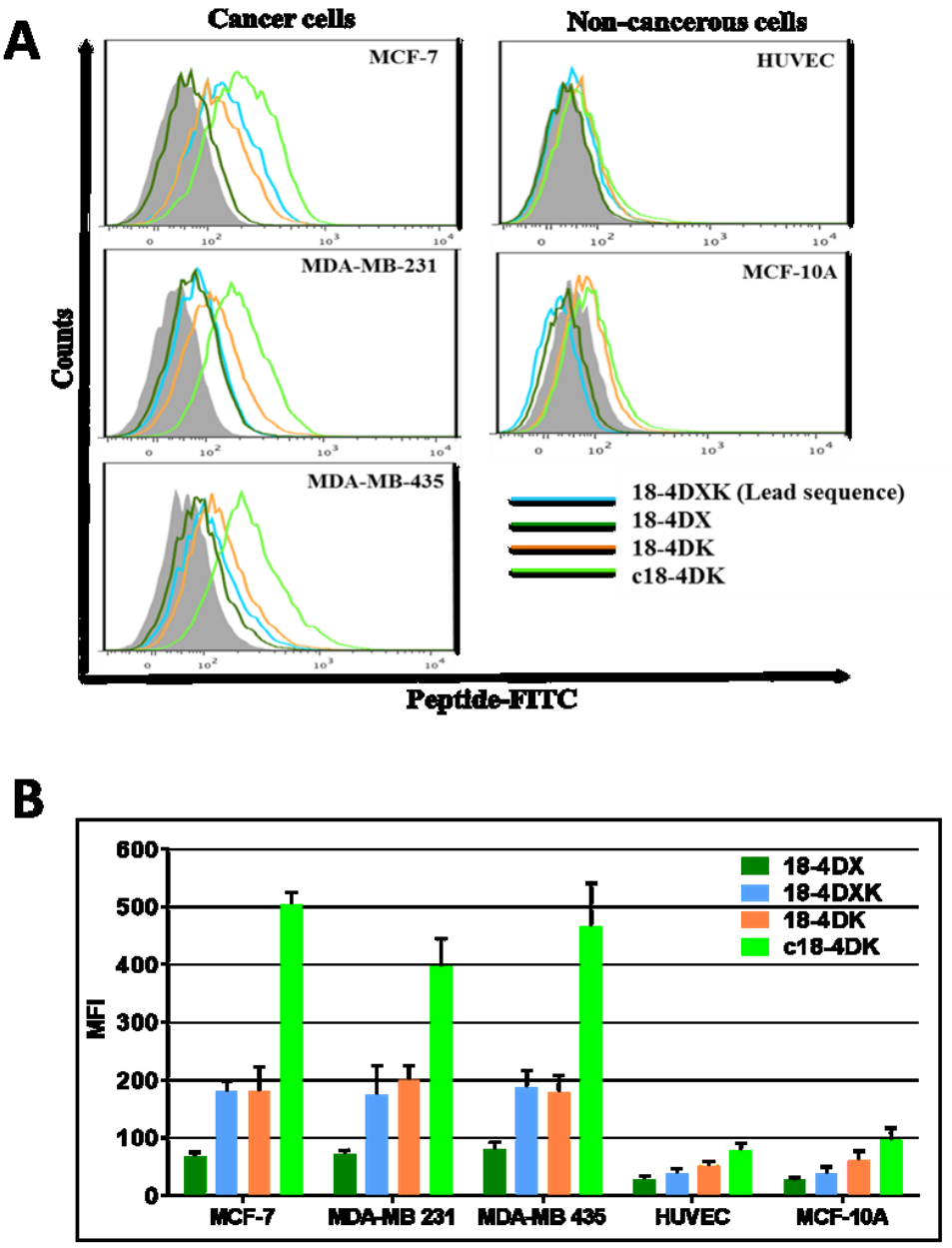


Figure 3.3: Peptide uptake by the breast cancer cells (Left) MDA-MB-435, MCF-7 and MDA-MB-231 and the control cells (right) HUVEC and MCF-10A measured using BD FACS Canto II flow cytometry. The peptides (10^{-6} mol/L or 1μM) FITC-18-4DXK (blue), FITC-18-4DX (green), FITC-18-4DK (orange), and

FITC-c18-4DK (light green) were incubated with the cells for 30 min at 37 °C. Auto-fluorescence of the cells is shown by shaded area. **A.** Shows overlays from one representative experiment for the uptake of peptides. **B**. Shows mean fluorescence intensity (MFI) of FITC positive cells from three independent experiments ±SD.

Previous studies have shown that cyclization reduces the hydrogen bonding and hydrodynamic radius in solution and increases the lipophilicity of the peptides and thereby enhances the membrane permeability, which could ultimately result in high cell uptake (74-76). In conclusion our preliminary results show that D-Lys8 substitution is important and beneficial for increasing specific binding of the peptide to the breast cancer cells and cyclization has further enhanced the specificity for the cell surface receptors. Based on the above results for peptide uptake using breast cancer cells, the peptide c18-4DK was selected for further studies.

3.3.1 Concentration-dependent uptake of peptides

As detailed in previous section, first we have identified best peptide analogue using comparative uptake study of 18-4DXK, 18-4DX, 18-4DK and c18-4DK. This study allowed us to select the best peptide in terms of selectivity and affinity for breast cancer cell lines. We found that c18-4DK showed highest uptake in breast cancer cell lines MDA-MB-435, MCF-7 and MDA-MB-231cells, while negligible binding to MCF-10A and HUVEC cells. At this point, a cyclic peptide with all L-amino acids, namely, c18-L (**Table 3.1**) was also included in the cell uptake study.

The concentration-dependent uptake study was carried out with the aim to check whether concentration of peptide also play a crucial role in uptake. Also using this assay we compared original peptide p160 with its first and second generation analogues designed and synthesized and published in Kaur lab (117, 119, 120). For this five peptides have been studied, namely p160, p18, 18-4DXK, c18-4DK and c18L. All the peptides were FITC labeled and studied at two different concentrations (1 μM and 10 μM). The overall data from **Figures 3.4, 3.5 and 3.6** showed that as the concentration of peptide was increased from 1 μM to 10 μM the magnitude of cell uptake was drastically increased.

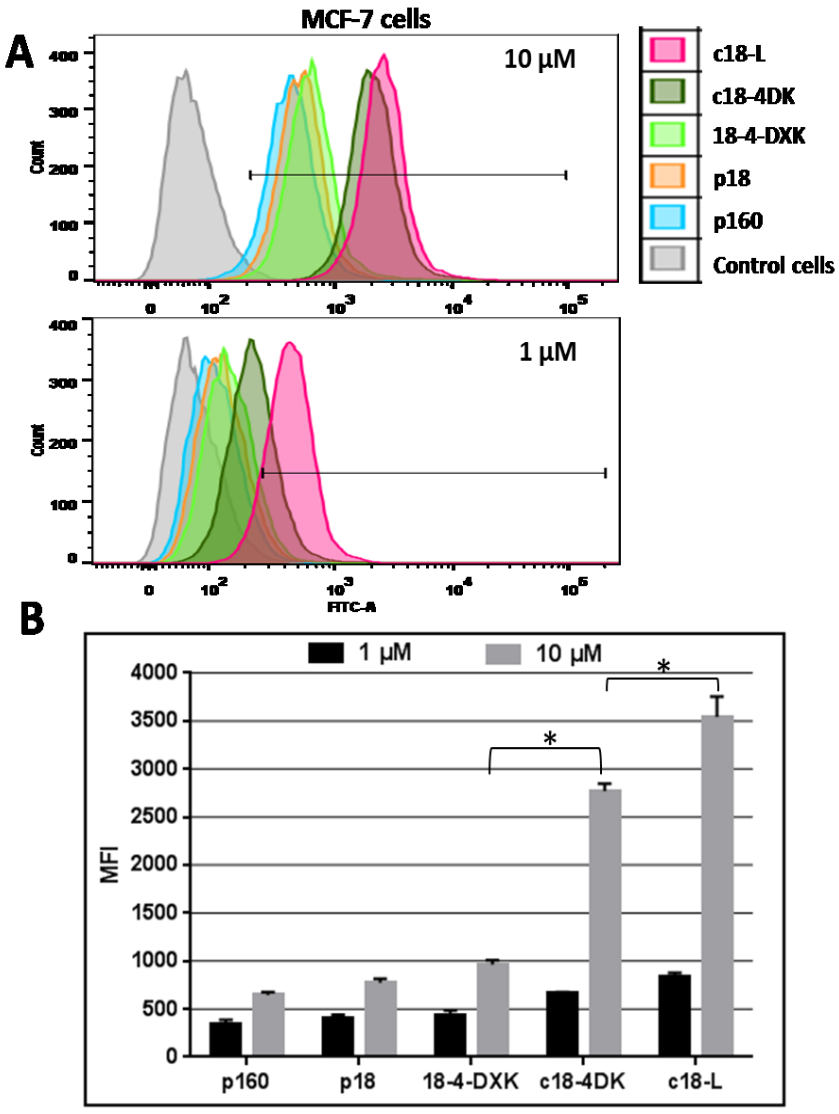


Fig. 3.4: Concentration-dependent uptake of peptides using MCF-7 cells. The MCF-7 cells were grown and incubated with 1 µM and 10 µM of respective peptide for 30 min at 37ºC and studied for uptake using flow cytometry. **A.** Shows overlays for the uptake of peptides at different concentrations. **B**. Shows mean fluorescence intensity (MFI) of gated FITC positive cells of three independent experiments ±SD. * Denotes statistically significant difference ($p<0.05$).

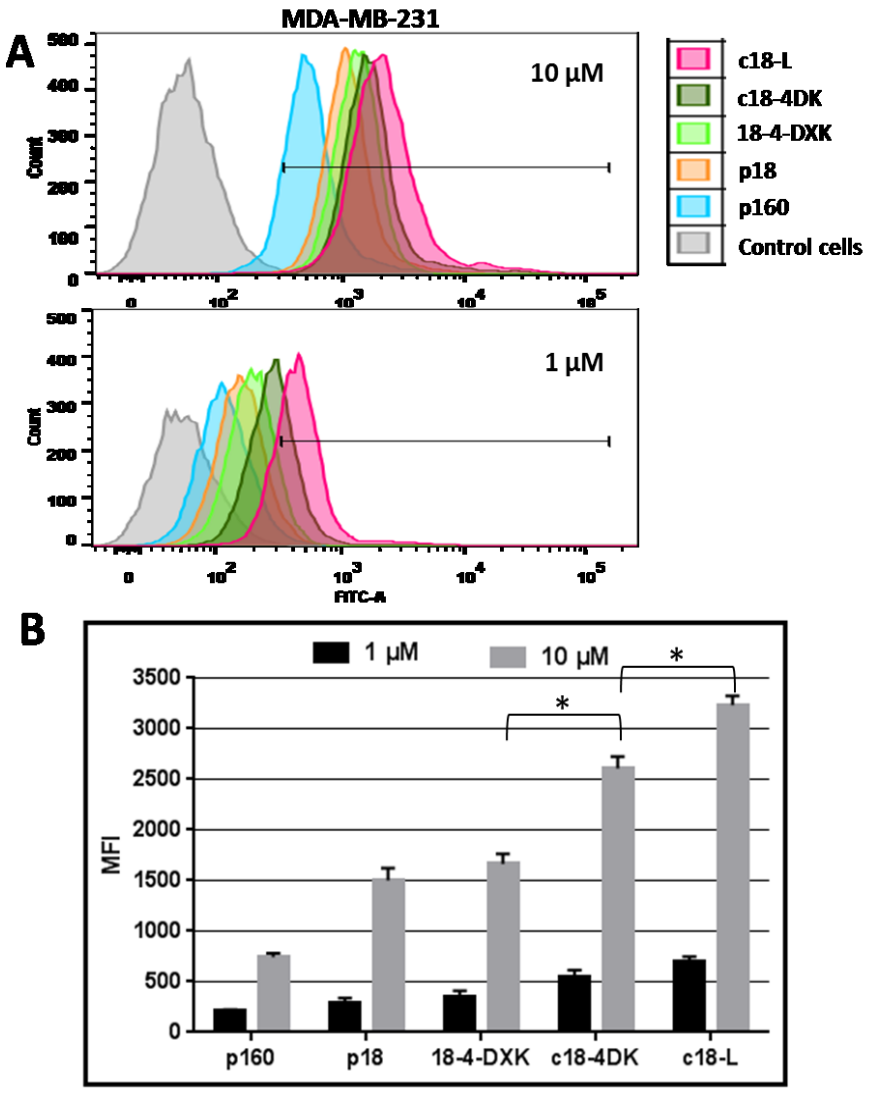


Fig.3.5: Concentration-dependent uptake of peptides using MDA-MB 231 cells. The MDA-MB 231 cells were grown in 12-well plates and incubated with 1 µM and 10 µM of respective peptide for 30 min at 37ºC and studied for uptake using flow cytometry. **A.** Shows overlays for the uptake of peptides at different concentrations (1 µM and 10 µM). **B**. Shows mean fluorescence intensity (MFI) of gated FITC positive cells from three independent experiments ±SD. * Denotes statistically significant difference ($p<0.05$).

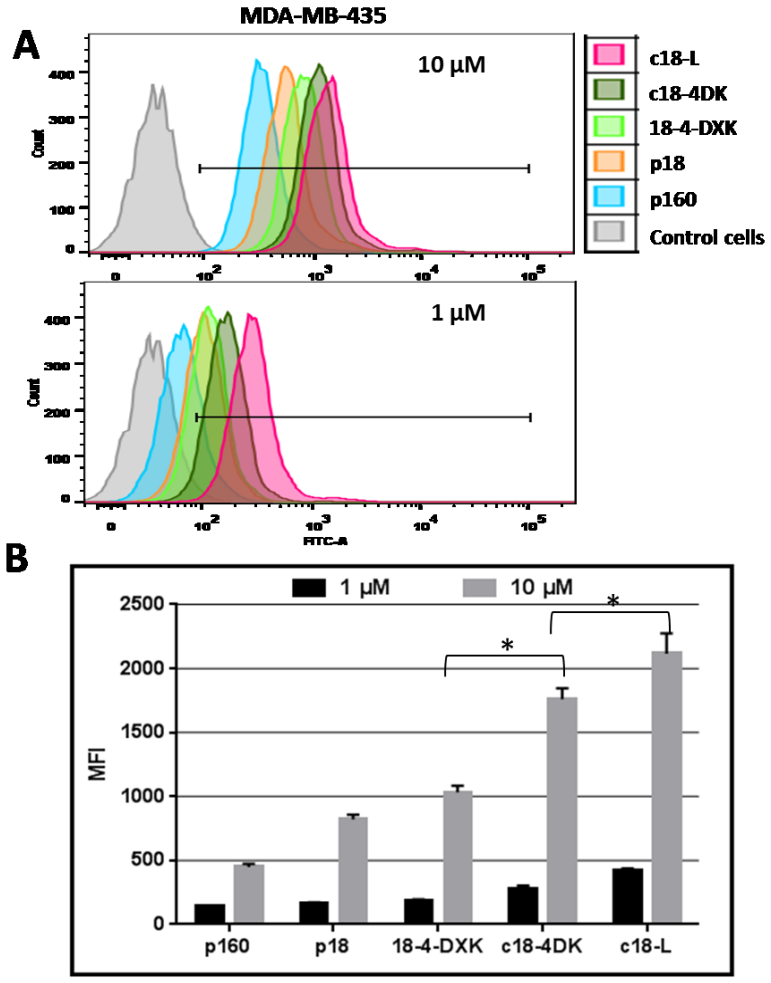


Fig. 3.6: Concentration-dependent uptake of peptides using MDA-MB 435 cells. The MDA-MB 435 cells were grown overnight in 12-well plates and next day cells incubated with respective peptide for 30 min at 37ºC and uptake was studied using flow cytometry. **A.** Shows overlays for the uptake of peptides at different concentrations (1 µM and 10 µM). **B**. Shows mean fluorescence intensity (MFI) of the cells gated for FITC from three independent experiments ±SD. * Denotes statistically significant difference ($p<0.05$).

The intensity of cell-associated fluorescence at 10 μM peptide concentrations was found to almost double or more than double when compared to the cell-associated fluorescence observed at 1 μM of peptide. This trend was observed for all breast cancer cell lines such as MCF-7, MDA-MB 231 and MDA-MB-435 cells used in our study. Peptide c18L showed the highest cell uptake (**Figure 3.4B, 3.5B and 3.6 B**). Overall the uptake was observed in the following order p160< p18<18-4DXK<c18-4DK<c18L.

Our results of concentration-dependent study showed that the cyclic analogs c18-4DK and c18L performed better than our previous published sequence 18-4DXK in binding to breast cancer cell lines (120). The mean fluorescence intensities (MFI) of c18-4DK and c18L were significantly different ($p<0.05$) from those observed for 18-4DXK sequence. Once again this trend was consistent with all of the breast cancer cells used in our study.

Furthermore, the comparative evaluation of different peptide analogues in one set of experiment allowed us to comprehensively compare the analogs for binding to breast cancer cells. As published previously from our lab, the results of concentration-dependent uptake study further confirm that peptide analogs p18 and 18-4DXK showed better uptake and binding to the breast cancer cells. The differences in uptake were more pronounced when peptides were used at higher concentrations. The data from concentration-dependent uptake also showed that c18L peptide shows higher affinity compared to c18-4DK peptide.

We have also evaluated these peptides for uptake using non-cancerous cell lines such as HUVEC and MCF-10A cells and experiments were carried out using same protocol as used for breast cancer cells (**Figure 3.7 A and B**). The results from these experiments showed that the peptides affinity to these cells is very low. The lower levels of uptake could be due to presence of very less putative receptors which are responsible for peptide uptake from these cells. Based on comparative evaluation of the MFI values obtained from MCF-7 and MDA-MB 231 cell with those of HUVEC and MCF-10A cells, we found that intensity of uptake was approximately 10 times more for breast cancer cells at 10 μM for c18-4DK and c18L peptides **(Figure 3.4B, 3.5B and 3.7B**).

Next the two promising peptides c18-4DK and c18L were evaluated for binding at 4 ºC at 10 μM concentration using MCF-7 and MDA-MB 231 cells (**Figure 3.8**). For binding assay the cells were kept on ice for 5 min and then FITC conjugated peptides dissolved in ice-cold growth media were added. Our results of binding at 4 ºC show that once the cells are metabolically rendered inactive the binding of peptides was drastically reduced compared to that observed with 37 ºC. These results are consistent with the previous studies from other groups (117, 118) showing drastic decrease in binding at lower temperatures.

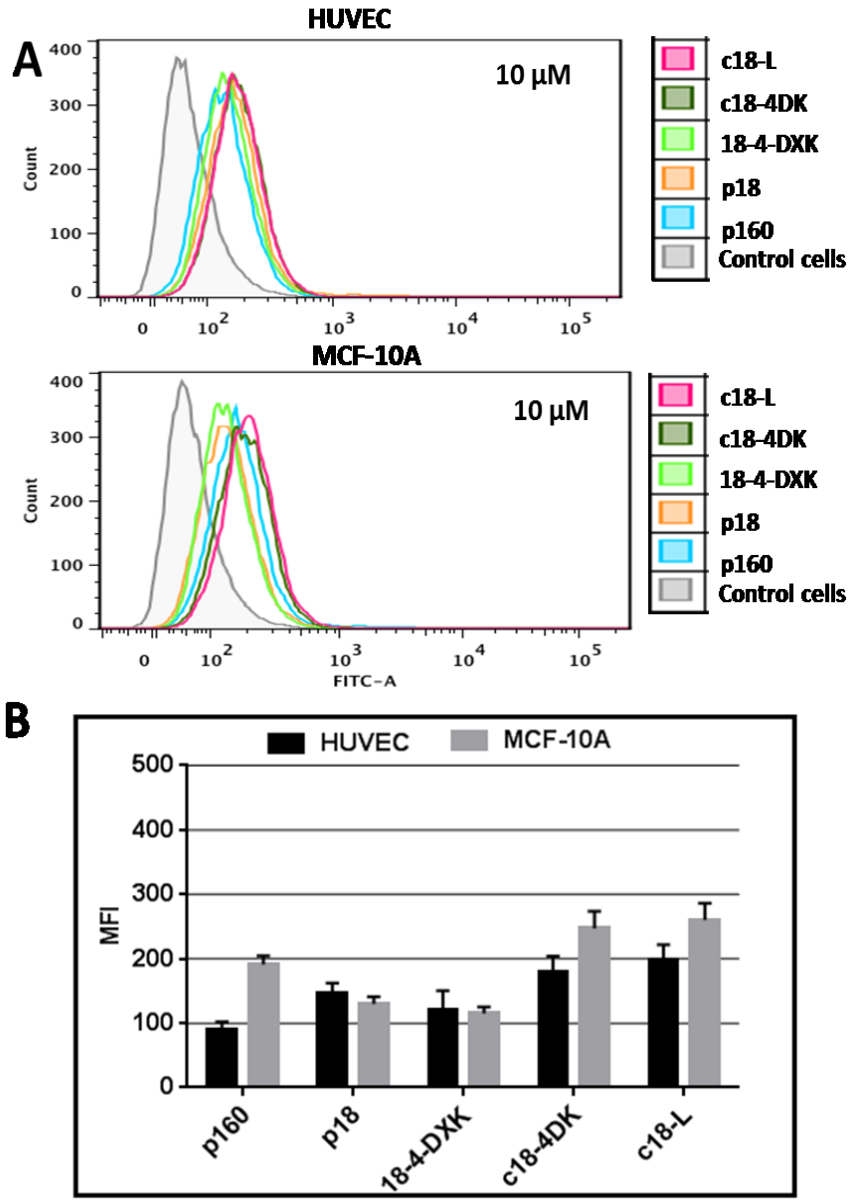


Fig. 3.7: Uptake of peptides using non-cancerous HUVEC and MCF-10A cells. The HUVEC and MCF-10A cells were grown in 12-well plates and incubated with respective peptide for 30 min at 10 µM concentration and uptake was studied using flow cytometry. Data was analyzed using FlowJo software **A.** Overlays showing uptake of the peptides at 10 µM concentration using HUVEC and MCF-10A cells. **B**. Shows mean fluorescence intensity (MFI) of three experiments ±SD.

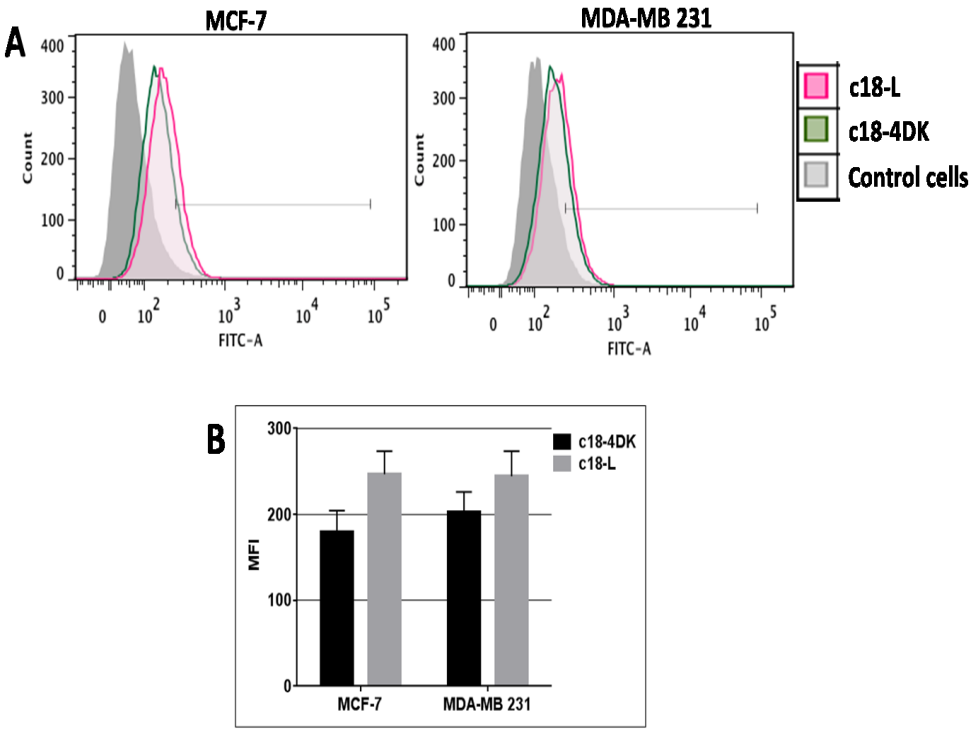


Fig. 3.8: Binding of peptides using MCF-7 and MDA-MB 231 cells. The MCF-7 and MDA-MB 231 cells were seeded and incubated overnight in 12-well plates and following day incubated with 10 µM of FITC labelled peptide analogues c18-4DK and c18L for 30 min at 4ºC and studied for binding using LSR-Fortessa flow cytometer. **A.** Overlays shown are from representative of one experiment out of three independent experiments performed at 10 µM peptide concentration. **B.** Shows mean fluorescence intensity (MFI) of three experiments ±SD

3.3.2 Trypsinization versus scraping

Trypsin is a serine protease, acts by cleaving peptide chains mainly at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline (124). It is used for numerous biotechnological processes. The process is commonly referred to as trypsin proteolysis or trypsinization, and proteins that have been digested/treated with trypsin are said trypsinized.

The comparative uptake study using trypsin and scraping as source for harvesting cells underlines the importance of harvesting methods for adherent cell lines like breast cancer cell lines used here for uptake studies using flow cytometry. We observed that different MFI values were obtained depending on the method of detachment of cells: cell trypsinization or scraping at 10 μM of peptide concentrations used for this study (**Figure 3.9, 3.10 and 3.11**). The MFI value obtained from scrapped cells was higher than that obtained with trypsinized cells and this trend was observed with all the peptides analogues studied. Also the MFI value of scrapped cells was overall significantly higher as compared to trypsinized cells and trend was irrespective of breast cancer cell line used. Overall we found that trypsinization of cells resulted in the reduction of MFI values from 10 to 30% when compared to scrapped cells (**Figure 3.11B, 3.10B and 3.11B)**.

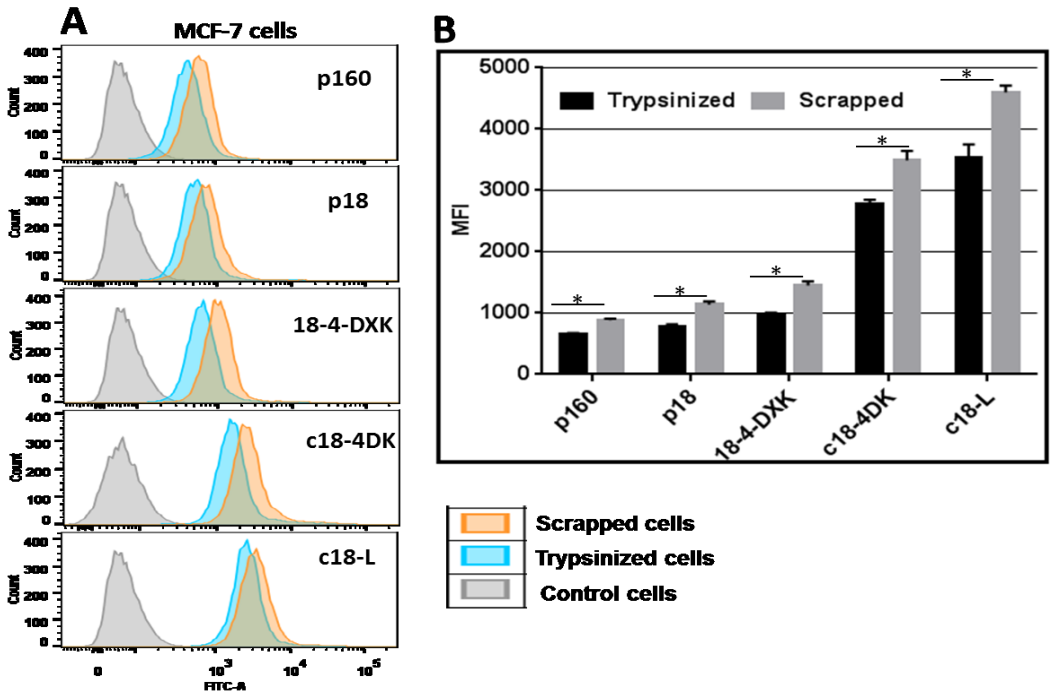


Fig. 3.9: Comparative evaluation of peptide uptake following trypsinization and scrapping of MCF-7 cells. The MCF-7 cells were incubated with 10 µM of indicated peptide for 30 min at 37ºC and after this cells were either chemically dislodged with trypsin or physically by scrapper. The processed cells were analyzed using flow cytometry and data was analyzed using FlowJo software. **A.** Overlays show for the comparative uptake profile of peptides subjected to trypsinization or scrapping at 10 µM concentration. **B**. Shows mean fluorescence intensity (MFI) from three independent experiments ±SD. * Denotes statistically significant difference ($p<0.05$).

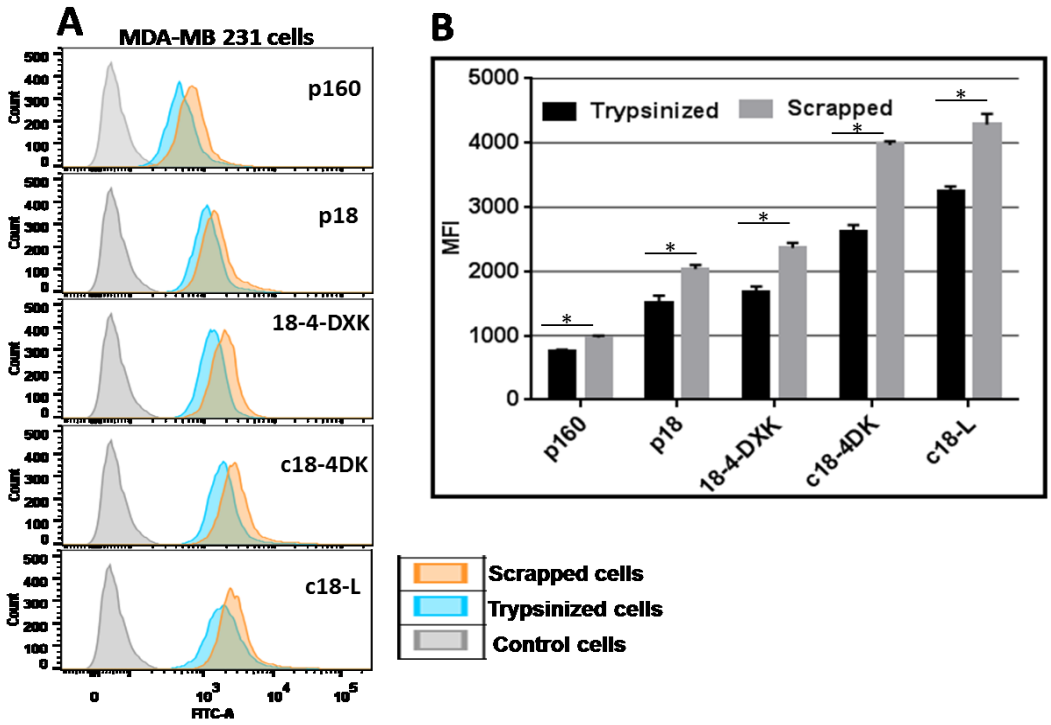


Fig. 3.10: Comparative evaluation of peptide uptake following trypsinization and scrapping of MDA-MB 231 cells. The MDA-MB 231 cells were incubated with 10 µM of indicated peptide for 30 min at 37ºC and after this cells were either chemically dislodged with trypsin or physically by scrapper. The processed cells were analyzed using flow cytometry and data was analyzed using FlowJo software. **A.** Overlays show for the comparative uptake profile of peptides subjected to trypsinization or scrapping at 10 µM concentration. **B**. Shows mean fluorescence intensity (MFI) from three independent experiments ±SD. * Denotes statistically significant difference ($p<0.05$).

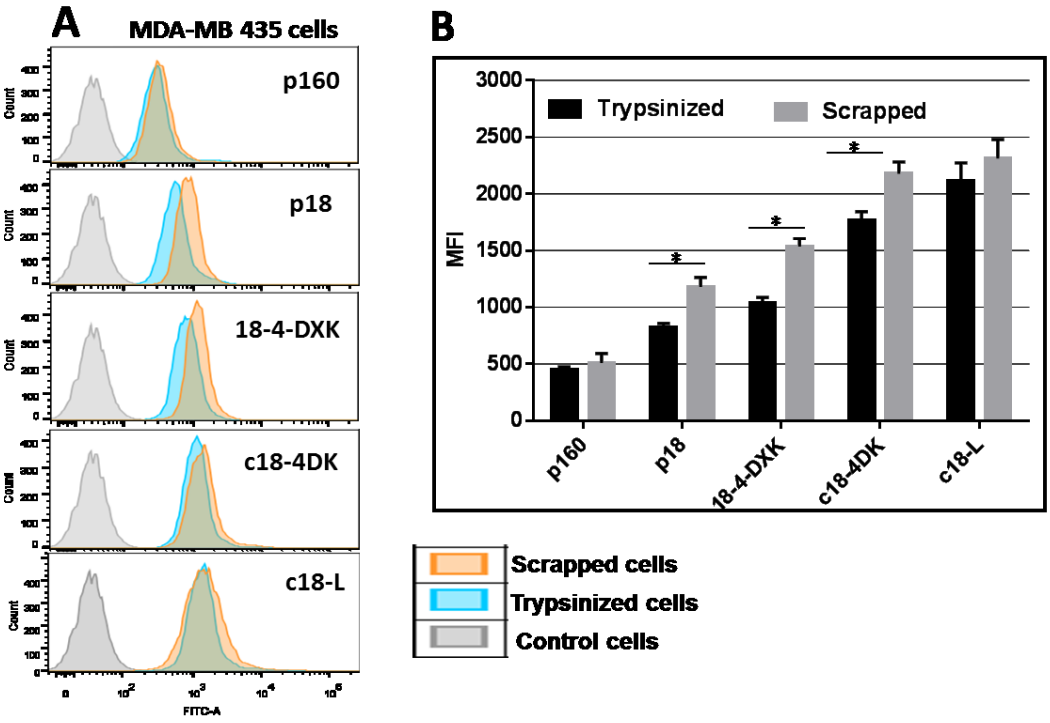


Fig. 3.11: Comparative evaluation of peptide uptake following trypsinization and scrapping of MDA-MB 435 cells. The MDA-MB 435 cells were incubated with 10 µM of indicated peptide for 30 min at 37ºC and after this cells were either chemically dislodged with trypsin or physically by scrapper. The processed cells were analyzed using flow cytometry and data was analyzed using FlowJo software. **A.** Overlays show for the comparative uptake profile of peptides subjected to trypsinization or scrapping at 10 µM concentration. **B**. Shows mean fluorescence intensity (MFI) from three independent experiments ±SD. * Denotes statistically significant difference ($p<0.05$).

These results suggest that, compared with trypsinization, the suitable detachment method for cell uptake studies is scraping. Trypsin has been shown to alter the integrity of cell surface proteins or receptors (125) and because of their enzymatic property it can work like razors and chop off the cell surface markers. The results showed that mechanical cell scraping provides the higher peptide uptake by the breast cancer cell lines, which might be because of the less damage to the surface protein of interest and thus changing the flow characteristics whereas trypsin was damaging the cell surface receptors.

3.3.3 Competitive uptake of peptide

Assuming whether the presence of an excess amount of free unlabelled peptide can engage the putative target receptor that bind preferentially to the peptide under study, cells were incubated with 50-fold higher concentrations of unlabelled peptide c18-4DK and then FITC- c18-4DK peptide conjugate was added. For this experiment we used breast cancer cell lines MCF-7 and MDA-MB-435 cells. The cells were incubated with FITC-labeled c18-4DK peptide (1 μM) in the presence of a 50 μM of unlabeled c18-4DK (**Figure: 3.12**). The cells were analyzed using flow cytometry and decrease in % (percent) FITC positive cells was calculated. It was observed that fluorescence has been significantly decreased when FITC labeled peptide is incubated with excess of unlabeled peptide ($p<0.05$). The unlabeled peptide resulted in the reduced positivity from 95% to 22% (up to ~76% decrease) for MCF-7 cell line and from 98% to 27% (~72% decrease) for MDA-MB-231 cell lines, suggesting specific binding of the peptide c18-4DK to the cancer cells. The results further direct us to conclude that uptake of peptides can be reduced when the putative receptor has been preoccupied with excess ligand.

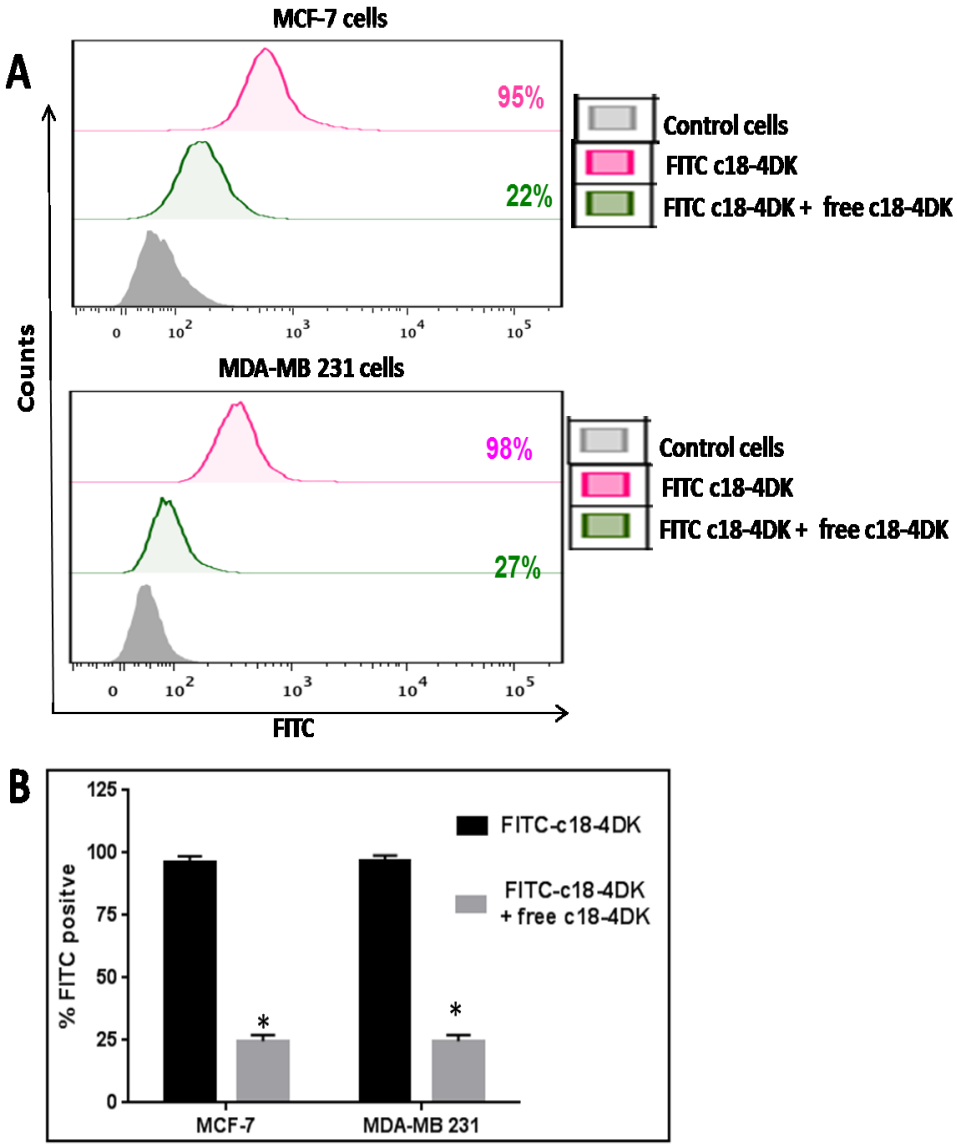


Fig. 3.12: Competitive uptake studies of c18-4DK peptide using MCF-7 and MDA-MB 231 cells. The MCF-7 and MDA-MB 231 cells were seeded in 12-well plates and grown overnight. Following day media was taken out and cells were pre-incubated with 50 µM of unlabelled c18-4DK for 15 min at 37ºC. Thereafter, the FITC conjugated c18-4DK was added at 1 µM and incubated for 30 min and cells were dislodged by trypsin treatment. The processed cells were analyzed using flow cytometry and data was analyzed using FlowJo software. **A.** Overlays show the comparative uptake profile of peptides subjected to uptake with or without free c18-4DK peptide. **B**. The bar diagram shows percent of FITC positive cells in the absence or presence of free c18-4DK from three independent experiments ±SD. * Denotes statistically significant difference ($p<0.05$).

3.4 **Proteolytic stability**

The proteolytic stability of the peptides c18-4DK (cWXEAAYQkFL) and c18L cWXEAAYQKFL) was explored. Susceptibility to proteolysis was compared with alpha sequence 18L (WXEAAYQKFL). Peptides (1 mM) were incubated with human serum at 37 °C and aliquots were removed at different time points. For these experiments 25% human serum was used. The samples were analyzed for any degradation product using reversed-phase HPLC. A linear gradient of 15-55% acetonitrile/water in 50 min with a flow rate of 2mL/min (Vydac C18 semi preparative column) was used and the absorbance of the eluting peaks was detected at 214 nm. The concentration of peptides and degradable products was measured by integrating the area under the curve and their identity was confirmed using MALDI-TOF mass spectrometry.

Figure 3.13 shows chromatograms of peptide c18-4DK incubated with human serum for 0, 6 and 24 h. Peptide c18-4DK was completely degraded at 24 h, giving a degradation product that appears after 12 min of incubation with human serum (**Table 3.2**). The degradation product of peptide c18-4DK showed mass of 972.6 g/mol. This was in accordance with previous study done by Kaur group with peptide 18 (120). The HPLC chromatogram of peptide c18L incubated with human serum at 0h, 4h and 6h are shown in **Figure 3.14**. More than 80% peptide degraded in the first 4 hours and after 6 hr no intact peptide was left suggesting complete degradation.

Table-3.2: The c18-4DK cyclic peptide sequence (cWXEAAYQkFL) was incubated with human serum for 0, 6h and 24 h at 37°C. The stability profile was analyzed using analytical RP-HPLC (Figure: 3.13). The degradation product of peptide c18-4DK after 12 min shows mass of 972.6.

Incubation Time (h)	Rt (min)	Peptide sequence	% intact peptide
0	28.1	c18-4DK (WXEAAYQkFL)	100
6	28.1	c18-4DK (WXEAAYQkFL)	64
24	28.1	c18-4DK (WXEAAYQkFL)	32
	12.2	Degradation product	-

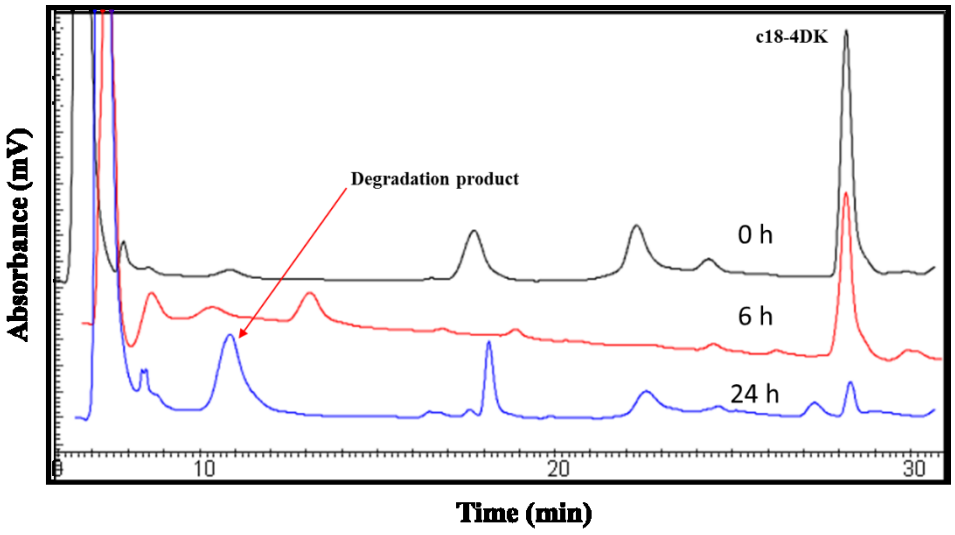


Figure 3.13: RP-HPLC chromatograms of peptides c18-4DK after incubation with human serum. Peptide was incubated with serum for different time intervals, namely, 0 h (black), 6 h (red) and 24 h (blue), prior to HPLC analysis. Peptides elute around 27-28 min. Degradation product appear around 12-13 min, and the remaining peaks are from the medium.

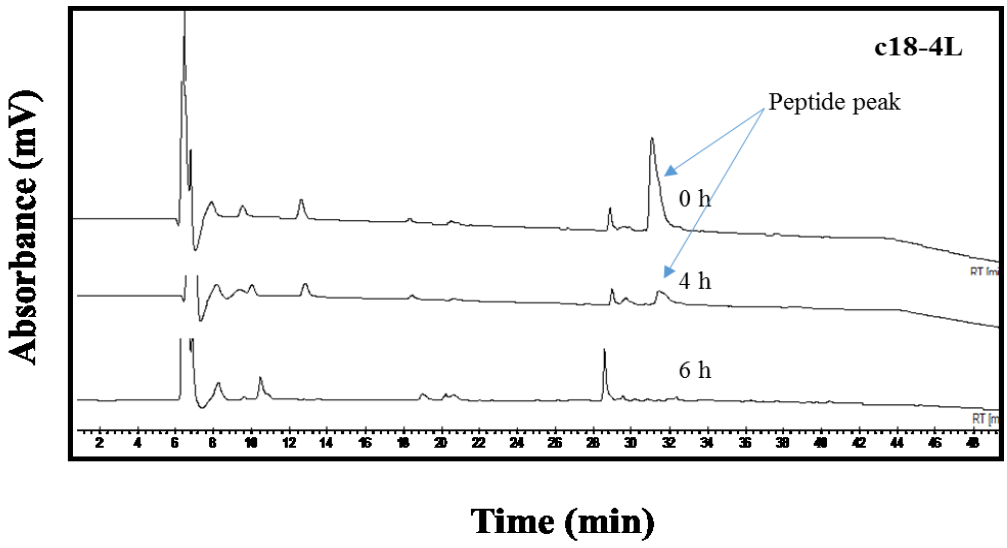


Figure 3.14: RP-HPLC chromatograms of peptides c18L after incubation with human serum. Peptide was incubated with serum for different time intervals, namely, 0 h, 4 h and 6 h, prior to HPLC analysis. Peptides elute around 30-31 min and the remaining peaks are from the medium.

To test the efficacy of human serum, an alpha peptide (18L) was incubated with human serum. The 18L peptide (WXEAAYQKFL) was completely degraded in 30 min giving a degradation product at 12-13 min. (**Figure: 3.15**). Blank solutions and the control (peptide 18L) were similarly treated for comparison.

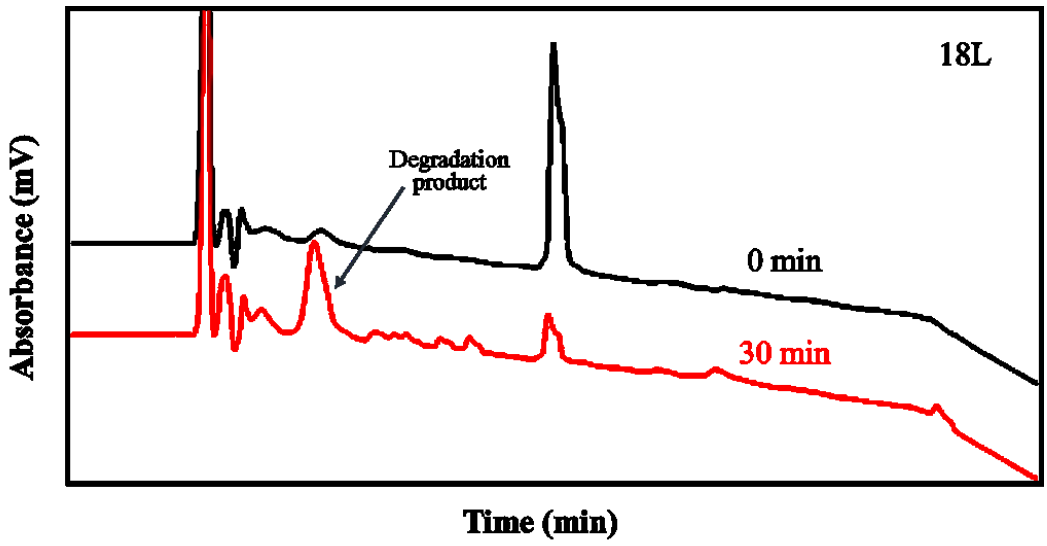


Figure 3.15: RP-HPLC chromatograms of peptides 18L after incubation with human serum. Peptide was incubated with human serum for different time intervals, namely, 0 h (black), 30 min (red), prior to HPLC analysis. Peptides elute around 22-23 min. Degradation product appeared around 12-13 min, and the remaining peaks are from the medium.

These results reveal that substitution of L-amino acids with the D-residues in the peptide sequence increase the proteolytic stability of the modified peptide relative to their α-peptide counterpart. Also, cyclization confers conformational restrictions which enhances the affinity and selectivity towards the target cells. These data reveal that the cyclic peptide c18-4DK provides substantial resistance to proteolysis which is essential for tumor drug targeting. In general, these results reflect that D substituitions are important for providing proteolytic stability to the peptides.

3.5 **General Conclusions**

Conventional cancer therapies have little or no specificity for tumor cells and normally result in severe side effects due to non-specific delivery of cytotoxic drug to healthy tissues. Therefore, it is desirable to improve the concentration of chemotherapeutics at the tumor site, thereby increasing the anticancer efficacy while reducing the associated systemic toxicity. The challenge to improve therapeutic efficacy of chemotherapeutic agents has resulted in the development of several types of tumor targeting ligands. These ligands have been shown to bind specific receptors present on the surface of cancer cells. In the tumor targeting approach, chemotherapy drugs can be actively targeted to tumor cells using tumor specific ligands such as peptides. Peptides have shown great promise as tumor targeting agent owing to their small size, lower immunogenicity, less toxicity and simplicity in design and synthesis but this potential is often overshadowed due to their low proteolytic stability. Therefore, in order to be used as drugs or ligands for targeted delivery, peptides need to be chemically modified. In this respect, substitution of natural amino acids (L) with the unnatural ones (D) or peptide cyclization are most common strategies that can be used to increase peptide stability, while maintaining the selectivity and affinity towards target tumor cells.

Recently many peptides have been identified by phage display technique and majority of them have been used for cancer diagnostic application and as imaging probes but their therapeutic application still remain unexplored. Using phage

display method a dodecapeptide p160 (VPWMEPAYQRFL) was identified and this peptide was shown to bind strongly to a range of neuroblastoma cell lines and a breast cancer cell line (117). Kaur lab has worked extensively on the synthesis of various analogues of p160 peptide for achieving better specificity towards breast cancer cells with and improved proteolytic stability (120, 121, 126).

In this thesis our objective was to engineer synthetic analogues of 18-4DXK, for enhanced affinity towards breast cancer cell lines while maintaining the optimum proteolytic stability profile. The data here show that three analogues of peptide 18-4DXK namely; 18-4DK, c18-4DK (D-lysine in the sequence) and c18L were designed and synthesized. These analogues displayed a significant increase in cell uptake and higher specificity for breast cancer cell lines such as MDA-MB-435, MDA-MB-231 and MCF-7, while relatively low binding to non-cancerous cells (HUVEC and MCF-10A). Furthermore, these analogues demonstrated selectivity and strong preferential binding to breast cancer cells compared to the lead sequence 18-4DXK and another analogue 18-4DX. The analogue 18-4DX (WxEAAYQKFL) where one labile residue was replaced with L-lysine and contain only D residue of nor-leucine (x), did not show enhanced binding to breast cancer cells as compared to parent peptide 18-4DXK. Interestingly, when the 18-4DK linear analog containing D isomer of lysine (k) was cyclized to obtain c18-4DK (cyclic WXEAAYQkFL), a further increase in the cellular uptake with the breast cancer cells was observed, while showing significantly lower uptake by

the noncancerous control cells. Furthermore, the analogue c18-4DK was also proteolytically stable compared to the linear or cyclic L-peptides.

The contemporary research findings show that cyclized RGD peptides can be used to effectively target tumors and as a result this strategy can be employed for successful imaging and treatment in preclinical models (102, 127). Additionally, structure-activity relationship (SAR) studies demonstrated that the receptor binding affinity and selectivity of RGD peptide can be enhanced by cyclization via linkers, such as thioether, disulfide, and rigid aromatic rings or other heterocycles (128, 129). An elegant study showed that, ACDCRGDCFCG (RGD-4C) with two disulfide bonds was at least 20-fold more potent than similar RGD peptides with a single disulfide bond, and 200-fold more potent than commonly used linear RGD peptides (130). Furthermore, other studies showed that cyclic RGD pentapeptide framework was able to increase the binding affinity and selectivity regardless of which amino acid is placed at position 5, viz., c(RGDfK), c(RGDfE), and c(RGDfV). Interestingly all of these three analogues showed similar binding affinity to integrin receptors (129). The use of tumor targeting peptides in general and RGD peptide in particular was validated with cyclic pentapeptide RGD analogue, Arg-Gly-Asp-Val or cyclo(RGDfV) also known as Cilengitide® (EMD 121974, Merck). It is a selective αvβ3 and αvβ5 integrin antagonist and demonstrated to show anti-angiogenic activity. However, despite promising phase I, and II clinical trials, this peptide was discontinued due to failure in phase-III clinical trials (131).

Based on the results of our studies it can be inferred that the substitution of natural amino acids (L) with the un-natural ones (D) increased the stability of peptides towards proteolytic enzymes when we compare the parent peptide 18-4DXK with the new synthesized analogues 18-4DX, 18-4DK, c18-4DXK and c18L. We found that conformationally constrained cyclic peptides c18-4DK and c18L analogues showed enhanced specificity for breast cancer cells. Furthermore, our results from concentration-dependent uptake study confirm that cyclization can influence the binding affinity towards breast cancer cells. Amongst the two peptide analogues, c18-4DK was found to maintain the proteolytic stability. The loss of resistance towards proteases for c18L might be because of the presence of the labile sites in the sequence. Therefore, it can be concluded that in the current study, cyclization of peptide augmented their binding affinity towards breast cancer cells, while not necessarily completely retaining the proteolytic stability. Therefore, considering c18-4DK as the best peptide, it was further studied in competition experiments to determine whether uptake could be reduced in the presence of free peptide which can potentially block the putative target receptors. The results of competitive blocking experiments using unlabelled c18-4DK with breast cancer cell lines MCF-7 and MDA-MB 231 cells potentially confirm the involvement of receptor mediated process in the uptake of these peptide analogues.

Moreover, the study examining the effects of scraping vs trypsinization on the magnitude of uptake cell trypsinization can lead to loss of cess associated fluorescence irrespective of peptides and cell lines used in the study. These

experiments suggest that the enzymatic activity of trypsin is possibly degrading some receptors on the surface of breast cancer cells and therefore use of trypsin should be minimized if not avoided while doing cell uptake experiments.

Conclusively our results show that substitution of natural amino acids (L) with the un-natural ones (D) increase the stability of peptides, while cyclization of peptide enhanced the affinity towards breast cancer cells. Overall these peptide analogues hold promising future and should be explored in preclinical trials for therapeutic and diagnostic applications.

3.6 **Future Directions**

In the current study, we have explored breast cancer targeting peptides, which can serve as favourable candidates for cancer therapy. First and foremost these peptides should be further investigated for the bio distribution and pharmacokinetic profile in tumor bearing mice.

Once these peptides show promising pharmacokinetic and bio distribution profile, it will be interesting to study whether cyclic peptides c18-4DK and c18L can show anti-tumor activity on their own or when conjugated with potential chemotherapeutic drug candidates. Furthermore, these peptides can be attached to the surface of promising drug delivery vehicles such as liposomes, nanoparticles, micelles to achieve selective delivery of drug to tumor vasculature.

Along with drug targeting the focus of contemporary research work is to use peptides as diagnostic moiety. Therefore, it will be interesting to see whether attachment of these peptides to fluorescent probes and radioactive compounds can help in imaging to tumors in animal models.

Furthermore, we speculate that these peptides are capable of binding to tumor cell-associated receptors. Therefore, it will be interesting to study the putative target receptors responsible for internalization of these peptides and the molecular mechanism involved.

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