Nanoscale Modification of Therapeutic Peptidomimetics to Improve Tissue Distribution and Cell Type Targeting

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In Chemistry
by

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Jadavpur University

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Dedicated To

Abba(RIP) and Ammi

CERTIFICATE FROM THE SUPERVISOR(S)

This is to certify that the thesis entitled ***"Nanoscale Modification of Therapeutic Peptidomimetics to Improve Tissue Distribution and Cell Type targeting."*** Submitted by Sri ***Israr Ahmed*** who got his name registered on ***9th February 2016 with index no. 21/16/Chem./24*** for the award of Ph.D (Science) Degree of Jadavpur University, is absolutely based on his own work under the supervision of ***Dr. Pradip Kumar Mahapatra and Prof. Siddhartha Roy*** and that neither his thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

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Abstract

TRTK-12 derived from the protein Cap Z $^{(265-276)}$ TRTLIDWNKILS binds to the S100B at an overlapping region with even tighter affinity than that of p53and thereby p53 is released from S100B-p53 complex and S100B is inhibited and thus the tumor suppressing activity of p53 is restored. Thus, a TRTK based pre-formed helical mimetic may have high enough affinity to disrupt p53-S100B interaction. Using the structure of S100B as a guide, those residues of the target peptide that are not interacting with S100B, but are still a part of the target sequence have been replaced by Aib.

Thus, starting from the unmodified p53 target sequence the design of a high bivalent peptidomimetic was achieved through progressive modifications. The augmented helicity of the bidentate branched peptidomimetic and enhanced protease resistance was verified. This bidentate branched peptidomimetic has strong affinity towards S100B.

The tissue distribution of bidentate branched peptidomimetics has been investigated by chelating it with radioactive Tc^{99} by tricarbonyl method and then measuring it by ϒ- counter. Result demonstrated the considerable distribution of the peptide in all the organs with an exception of brain and renal excretion of 68%.

In order to design a peptidomimetic which can be used to assay KLF1-DNA interaction, a conformationally-constrained helix of 29-mer derived from the KLF1 protein was designed and synthesized. The interaction of peptide with that of the designed DNA was measured which shows it was found that the peptide interacts with the DNA with a binding constant (K_D) value of 276±11.2nM. In order to determine the specificity of the KLF1 peptide with the specified DNA, a random DNA is selected and its binding isotherm with the random DNA is studied by anoisotropic method using fluorimeter and no binding affinity is found with random DNA indicating that it has a very high degree of specificity with the selected DNA.

A cyclic peptidomimetic which can function as peptide-based inhibitor of HCV RNA virus was designed and synthesized including one linker AHX was synthesized. This peptide was attached with the synthetically modified berberine derivative. The linear form of this peptide is also coupled with CSP nanoparticle which will help the peptide not only to penetrate the cell but will also serve as nuclear localizing sequence. Further the peptide is attached with CSP nanoparticle and is characterized by measuring zeta potential and elemental analysis

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Israr Ahmed

Abbreviations

A°: Angstrom

ADP: Adenosine 5’-diphosphate

ATP: Adenosine 5’-triphosphate

Ahx: 6-Aminohexanoic Acid

Aib: Aminoisobutyric Acid

bp: Base pair

BSA: Bovine Serum albumin

CD: Circular Dichroism

CSP: Carbon Sphere Particle

CTD: C-terminal domain

Da: Dalton

DEAE: Diethylaminoethyl

DMF: Dimethyl Formamide

DMSO: Dimethyl sulphoxide

DNA: Deoxyribonucleic Acid

EDTA: Ethylenediaminetetraacetic acid

FITC: Fluorescein isothiocyanate

HPLC: High Performance Liquid Chromatography

kb: Kilobase pairs

kCal: Kilo calorie

Kd: Dissociation constant

kDa: Kilo Dalton

M: Molar

Min: Minute

mM: Millimolar

M: Micromolar

nM: Nanomolar

nm: Nanometer

NTD: N-terminal domain

PAGE: Polyacrylamide gel electrophoresis

RNA: Ribonucleic Acid

RRM: RNA Recognition Motif

SDS: Sodium dodecyl sulfate

TFA: Trifluoroacetic acid

Tris: Tris(hydroxymethyl)aminomethane

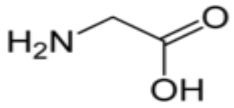
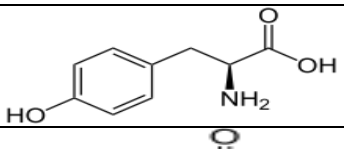
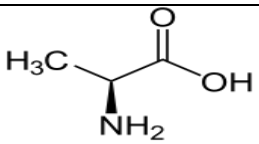
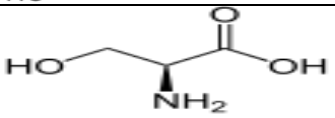
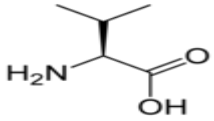
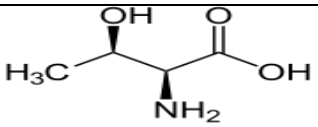
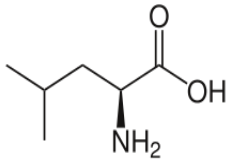
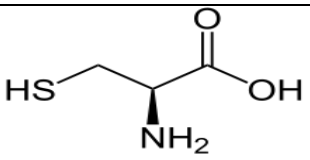
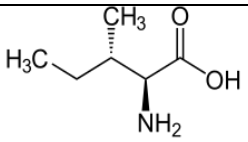
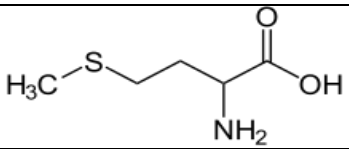
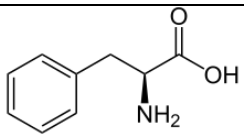
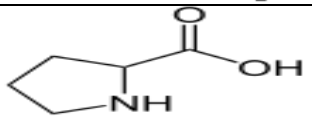
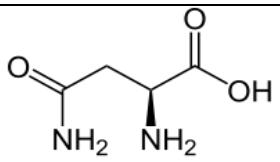
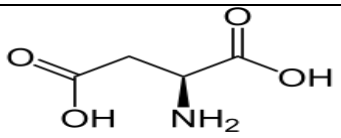
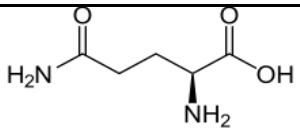
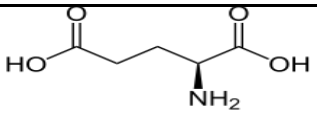
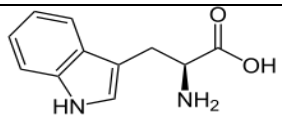
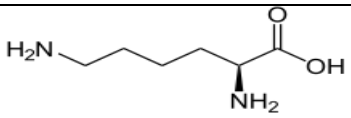
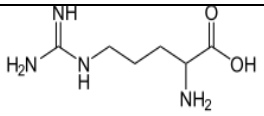
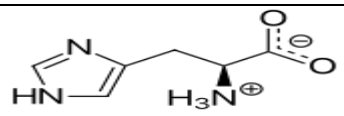
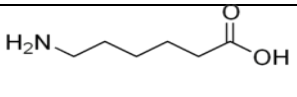
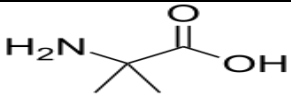
TSS: Transcription Start Site

UV: Ultraviolet

List of Amino Acids with their abbreviations:

Name of the Amino Acid	3-Letter Abbreviation	Single Letter Abbreviation
Glysine	Gly	G
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Phenylalanine	Phe	F
Asparagine	Asn	N
Glutamine	Gln	Q
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Serine	Ser	S
Threonine	Thr	T
Cysteine	Cys	C
Methionine	Met	M
Proline	pro	P
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E
Lysine	Lys	K
Arginine	Arg	R
Histidine	His	H
6-Aminohexanoic Acid	Ahx	-
Aminoisobutyric Acid	Aib	B

Structure Of Amino Acids

Amino Acid	Structure	Amino Acid	Structure
Glysine		Tyrosine	
Alanine		Serine	
Valine		Threonine	
Leucine		Cysteine	
Isoleucine		Methionine	
Phenylalanine		Proline	
Asparagine		Aspartic Acid	
Glutamine		Glutamic Acid	
Tryptophan		Lysine	
Arginine		Histidine	
6-Aminohexanoic Acid		Aminoisobutyric Acid	

CHAPTER 1

GENERAL INTRODUCTION

Proteins which are made up of one or more polypeptide chains are the most common material found in the cell. These are not merely rigid lumps of material and consist of a precise and specific amino acid sequence that permits it to acquire a particular three-dimensional shape commonly known as conformation through a process called protein folding. Their chemical events are exhibited by the mechanical motion of their precisely engineered part. Indeed, all proteins stick or bind to other molecules. In some cases, their binding is very tight in others it is weak and short-lived. But the binding is highly specific in nature indicating that each protein molecule can bind or interact with usually one or a few proteins out of the many different types it encounters. The substance that binds with a protein, whether it is an ion or a small organic molecule, or a macromolecule, is usually a ligand for that protein.

Proteins play many critical roles in the body. They do most of the work in cells and are required for the structure, function, and regulation of the body's tissue and organs. From the very beginning, protein being an undetectable and unavoidable part of biology, its roles are highly appreciated in all segments of biology particularly cell biology, molecular biology, structural biology, biochemistry, and biophysics. The individual protein present in a biological system is not a matter of interest unless it involves itself in protein-protein interactions (PPIs). Dysregulation of these PPIs exhibits serious threats in the form of diseases of which tumors are the most significant threat to human life. As shown in the figure 1.1, normally one protein attaches with another or many proteins via an electrostatic interaction or by hydrophobic interaction or hydrogen bonding interaction to form complexes that act as molecular machines exhibiting very complicated physiochemical dynamics which helps us to understand their biological functions at both cellular and systems levels **(De Las Rivas and Fontanillo 2010).**

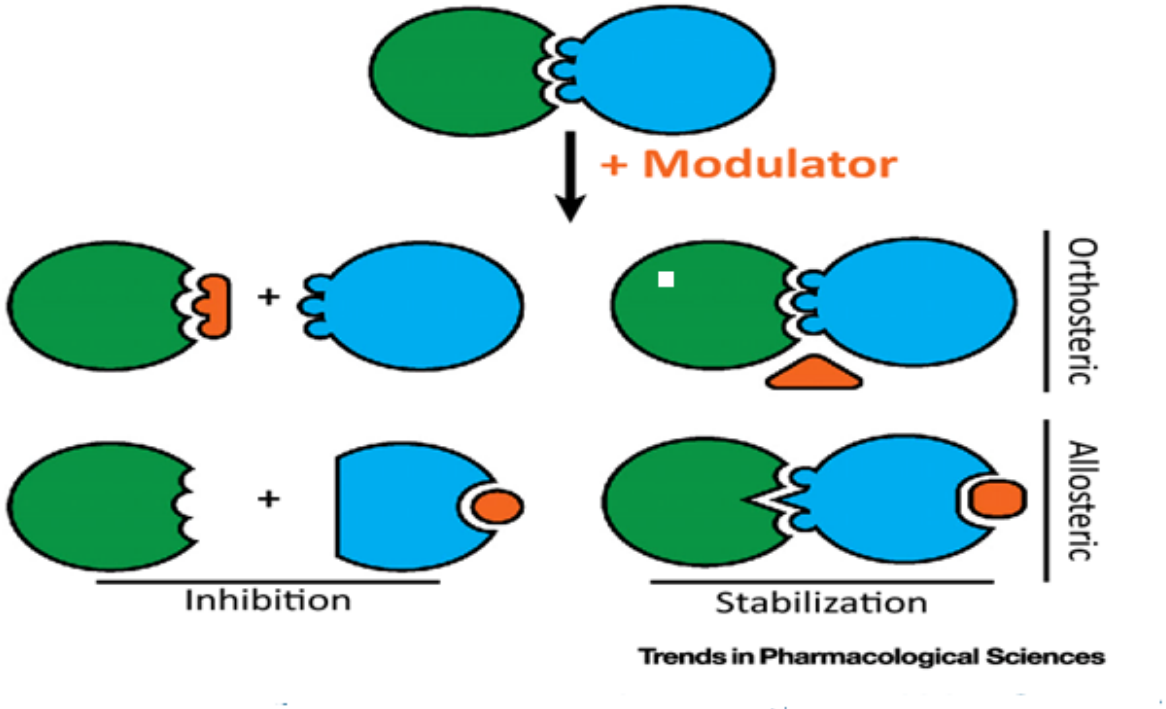


Figure 1.1: Systematic targeting of protein-protein interaction (Modell, Blosser et al. 2016)

1.1. Protein-protein interaction (PPI)

Proteins can act as independent molecules and therefore it interacts with other proteins to form functional complexes **(Miura and Imaki 2008, Alanis-Lobato, Andrade-Navarro et al. 2016)**. The formation of protein complexes greatly increases protein diversity. There is an enormous number of potential combinations of protein-forming complexes. Therefore, genomic information is inadequate to ascertain directly what kind of protein complexes can be formed physiologically.

Protein-protein complexes are often the most potent players in cellular processes and play a key role in the prediction of protein targets and the drug ability of molecules. The high specificity of interaction established between two or more protein molecules ensures many phenotypes, e.g. human ailments, are underpinned by one or few such interactions that include hydrophobic interactions, electrostatic forces, and hydrogen bonding **(Rao, Srinivas et al. 2014)**. Studying PPIs is becoming one of the major objectives of systems biology. These interactions between the residues and the side chains are of non-covalent in nature and the basis for PPI and as well as protein folding and protein assembly **(Ofran and Rost 2003)**. It has been revealed that proteins rarely act as an independent and isolated species while performing its function in a biological system **(Yanagida 2002)**. It has been observed that the majority of proteins, that is more than 80% of the protein, do not act alone but in complexes **(Berggård, Linse et al. 2007)**. An analysis of target proteins suggests that proteins involved in certain cellular processes are exhibiting their interactions with other proteins in many situations **(von Mering, Krause et al. 2002)**. PPIs are very important in order to figure out the protein functions within the cell. It helps to expedite the modelling of the functional pathways in order to exemplify the molecular mechanism of cellular processes. To understand the biochemistry of the cell, the characterization of protein complexes in a given proteome is needed **(Phizicky and Fields 1995, Zhang 2009)** . When a protein interacts with others, then there exist certain functional objectives which can be established by several ways. The main characteristic properties of PPI have been represented **(Phizicky and Fields 1995)** as follows :

PPI can

a) Inactivate or suppress a protein.

b) Construct a new building site for small effectors molecule.

c) Change the specificity of a protein for its substrate through interaction with different boundary proteins.

d) Serve a regulatory role in either upstream or downstream level.

e) Modify the kinetic properties of enzymes

f) Act as a general mechanism to allow for substrate channeling.

1.2. Peptidomimetics

A peptidomimetic is a molecule bearing an identifiable resemblance to a peptide that as a ligand of a biological receptor can mimic the effect of a natural peptide. Peptidomimetics are basically designed to mimic the essential elements of a natural peptide or protein in 3D space that act as a small protein-like chain and which retain the ability to interact with the biological target and produce similar biological effects. When the existing peptide is modified or a similar system is designed that mimics the peptide results in the formation of a typical peptidomimetic. Irrespective of this approach, the altered chemical structure is designed to adjust molecular properties such as stability or biological activity advantageously. This helps in the development of drug-like compounds from an existing peptide. This modification involves changes to the peptide that will not occur naturally. For example, anticancer peptidomimetics can bind to the target protein in order to induce cancer cells into a form of programmed cell death called apoptosis by mimicking key interactions that activate the apoptotic pathways in the specified cells. This shows that peptidomimetics can play a vital role in the treatment of various types of

cancers **(Li, Thomas et al. 2004)**. The imitation that can be done in a peptide to get a peptidomimetic can be done by mimicking either the side chain or the main chain or both. In addition, amino acid extension, deletion or substitution, and other potent backbone modifications are the most recent techniques.

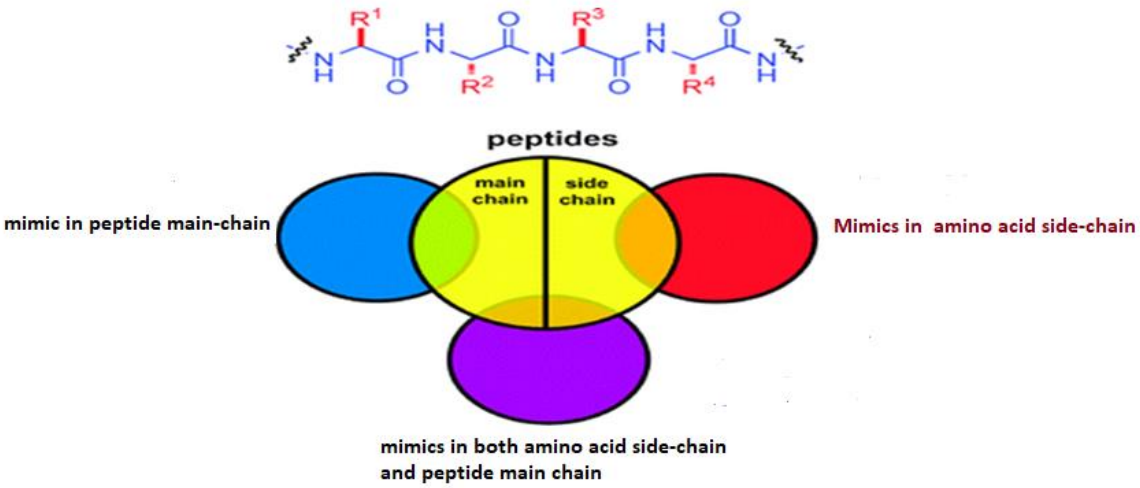


Figure 1.2: Mimics containing peptide bond. (De Las Rivas and Fontanillo 2010)

Peptides containing structural elements beyond 20 genetically encoded amino acids are peptidomimetics. These peptidomimetics have acquired an immense biological interest and therefore are in high demand in the current era. Peptides containing phosphorylated or glycosylated residues have been specifically emphasized as the incorporation of side chain phosphorylation in serine and in threonine is really a challenge in Solid phase peptide synthesis (SPPS). This is because the phosphate group is degraded by strong acid and is lost in the base by the β-elimination process. The useful and commercially available derivative of serine and threonine are Boc-Ser ($PO_3phenyl_2$) and Boc-Thr ($PO_3phenyl_2$) whereas hydrogenolysis by hydrogen fluoride (HF) cleaves the peptide/resin removing the phenyl group. This difficulty can

be avoided by using Fmoc-Ser(PO_3BZL,H) and Fmoc-Thr(PO_3BZL,H) with some care ***(OTVOS Jr, ELEKES et al. 1989, Wakamiya, Saruta et al. 1994***).

Another option for peptide resins that are built by Fmoc chemistry including unprotected serine and threonine side chains be subjected to global and post-assembly phosphorylation ***(Kitas, Perich et al. 1989)***. As far as the side chain protection of tyrosine is concerned it is less decomposed by strong acid and is not at all base labile. Thus SPPS has been used for the incorporation of Fmoc-Tyr(PO_3methyl$_2$) ***(PERICH and REYNOLDS 1991)***, Fmoc-Tyr(PO_3tBu_2) ***(Ottinger, Shekels et al. 1993)***, Fmoc-Tyr(PO_3H_2) ***(Zardeneta, Chen et al. 1990)*** and Boc-Tyr(PO_3H_2) ***(Cudic and Burstein 2008).***

The methodology has been developed for site-specific incorporation of carbohydrate molecules during the chemical synthesis of peptides. The mild condition of Fmoc chemistry has been preferred over Boc chemistry for glycopeptide synthesis as repetitive acid treatment is detrimental to sugar linkage. The successful incorporation of glycosylated entity has been performed in the side chain of Fmoc Ser, Thr, 5-hydroxylysine(-Hyl) and Asn***(OTVOS Jr, ELEKES et al. 1989)***. The side chain glycosyl is normally hydroxy group protected by either benzoyl or by acetyl group whereas some SPPs is successful with no hydroxyl group protection with glycosyl group ***(Sjölin, Elofsson et al. 1996)***. Prior to glycopeptide resin cleavage deacetylation or debenzoylation are performed with hydrazine/methanol ***(Tam, Wu et al. 1991***). In SPPS disulfide bond formation can be achieved by air, $K_3[Fe(CN)_6]$, dithiobis(2-nitrobenzoic acid) or by direct deprotection/oxidation of Cys(acetamidomethyl) residues using thallium trifluoroacetate or I_2 or by direct conversion of Cys(acetamidomethy) residue by thallium trifluoroacetate. There are a large number of reagents through which disulfide bond formations

are mediated in solution the most simple of which is molecular oxygen in DMSO ***(Kates, Solé et al. 1994)***.

1.3. Classification of peptidomimetics

Peptidomimetics have been classified into various types as follows:

(a)Type 1 peptidomimetic or Pseudopeptides: - In these peptides, the peptide backbone containing the functional groups that are in close contact with the binding site of the receptor are retained. The short portion of the secondary structure of the peptide is altered, making it a mimic of, for example, p-turn. This class has been used to generate lead compounds. Considering the design from substrate/product mimics of the peptide bonds in product state or in transition state includes many early-stage protease inhibitors that are designed for the enzyme catalyzed reactions.

(b) Type II peptide or functional mimetics: Molecular modelling and High Throughput Screening (HTS) etc. were used to synthesize these peptidomimetics. These are basically non-peptide small molecules that bind to a peptide receptor. These are normally structural analogs of the natural peptide. The characterization of the binding site of the antagonist and endogenous peptide by site-directed mutagenesis indicates that in the case of antagonists for a large number of receptors, binding subsites are different from that used by the parent peptide. As a result, functional mimetics may not mimic the structure of the peptide.

(c) Type III peptidomimetics or topographical mimetics: - These are basically the topographical mimetic of the peptide possessing novel templates positioned in peptide scaffold containing the essential group but seem to be unrelated to the original peptide. In order to

compare with the structure of the original peptide and with the heterocyclic nonpeptide inhibitor complexes, many type III peptidomimetic protease inhibitors have been characterized using x-ray. This structure determination revealed that alternate scaffolds can represent side chains so that they interact with the protein in the same fashion as that of the parent peptide.

(d) Type IV peptidomimetics or non-peptide mimetics: - Group Replacement Assisted Binding (GRAB) technique of drug design is used to synthesize this type of peptide. This structure is likely to share structural and functional features of type-I peptidomimetics but in contrast to type-I it generally binds tighter to an enzyme.

1.4. Peptidomimetics as therapeutic agents

Since a peptide binds to specific cell surface receptors or ion channels transmitting their intracellular effects, therefore peptides are recognized as highly selective and efficacious signaling molecules. A large population of peptides, nearly 7000, have been discovered so far exhibiting their crucial roles in humans' physiology including their action as ion channel ligands, growth factors, hormones, and neurotransmitters.

A novel therapeutic peptide synthesis includes its design as an excellent starting point as it represents a high degree of safety, efficacy, and tolerability profile in human beings. Due to these unique properties, peptides may succeed where small organic molecules fail as therapeutic agents. As far as the production complexity and cost are concerned, peptide therapeutics may exhibit easier production techniques but poorer cost comparison to that of small organic molecules. The efficacy of therapeutic peptide is balanced by its intrinsic weaknesses such as poor physical and chemical stability and short circulating plasma half-life.

These limitation needs to be mentioned when using therapeutic peptides. In the emerging opportunities and future direction in the peptides field, these limitations were partially resolved by multifunctional and cell-penetrating peptides, as well as peptide drug conjugate and technologies focusing on alternate routes of its administration **(Fosgerau and Hoffmann 2015).** A brief analysis of weakness, threats, strength and opportunities in using unmodified peptides as therapeutic agents are as follows:

Weaknesses:

- Short half-life and fast eliminations.
- Low membrane permeability.
- Chemically and physically unstable.
- Tendency for aggregation.
- Usually not orally available.
- Prone to hydrolysis and oxidations.

Threats:

- Increasing safety and efficacy requirements for novel drugs.
- Immunogenicity.
- New advancement in genomics, proteomics and personalized medicine.
- Price and reimbursement environment.

Strengths:

- Good efficacy, safety and tolerability.

- Standard synthetic protocols
- Predictable metabolism.
- High selectivity and potency.
- Lower attrition rate.
- Shorter time to market.

Opportunities:

- Alternative delivery route beside parenteral.
- Multifunctional peptides and conjugates.
- Discovery of new peptides including fragmentation.
- Formulation development.
- Focused libraries and optimized designed sequences.

1.5 Peptidomimetics in the treatment of cancers

At present time the cardiovascular disease is assumed to be the most serious and critical among the various ailments of human beings as it causes major casualties. But in recent future the mortality rate of cancer is supposed to succeed over cardiovascular disease. As per an estimation about 7 million peoples have to lose their lives every year suffering from cancer and its related diseases and 16 million cases are of suffering from cancer all around the world **(Jemal, Bray et al. 2011, Smith and Rennie 2014).**

Cancer is a deadly disease which is characterized by uncontrolled cell division and its unique ability to these cells to invade other tissues leading to the formation of tumor mass, vascularization and metastasis (spread of cancer to other part of the body)**(Vogelstein and**

Kinzler 2004). Growth of new blood cell from pre-existing blood cell is commonly known as angiogenesis which is anormal and vital process in cancer growth and development, it is assumed to be a fundamental step in the transition of tumor from a dominant state to a malignant one **(Folkman 1995).** The use of proteins, monoclonal antibodies (mAB) and peptides are the most common option that are in frequently used in the treatment of cancer. In comparison to peptide monoclonal antibodies and large protein ligands have two limitations one is the poor delivery to tumors due to their large size and the another one is dose limiting toxicity to the liver and bone marrow due to nonspecific uptake into the reticule endothelial system. The use of such macromolecules had therefore been restricted to either vascular target present in the luminal side of tumor vessels endothelium and hematological malignancies **(Allen 2002, Borghouts, Kunz et al. 2005).**

Small size, ease of synthesis and modification tumor penetrating ability and good biocompatibility are the advantages of using peptides as therapeutics in treatment of cancers **(Marschütz, Zauner et al. 2002).** Since many years peptide have been evolved as the most promising therapeutic agent in the treatment of cancer, diabetes and cardiovascular diseases and the application of peptide in the variety of other therapeutic area is growing very rapidly. Nearly 60 peptides drugs have been approved so far as therapeutic that are being utilized in the treatment of various cancers till now **(Ko, Liu et al. 2011).** In the treatment of various cancer peptide can be utilized in a number of different ways. The common strategies that help to employ peptide in the treatment and management of cancers includes using peptide directly as drug (e.g., as angiogenesis inhibitors), tumor targeting agent that carry cytotoxic drug and radionuclide's (targeted chemotherapy and radiation therapy) vaccines and hormones.

Different possible cancer treatment options using peptides are summarized in the figure 1.3.(Thundimadathil 2012)

1.6 Cell Penetrating peptides

For the greater understanding of disease at the molecular level a large number of potential therapeutic targets are being provided, which are increasing steadily. The challenge is to develop suitable ligands **(Frankel and Pabo 1988)**. Progress in drug development based on classical small molecules has been slow and often fails to target protein-protein interaction with high specificity in many cases.

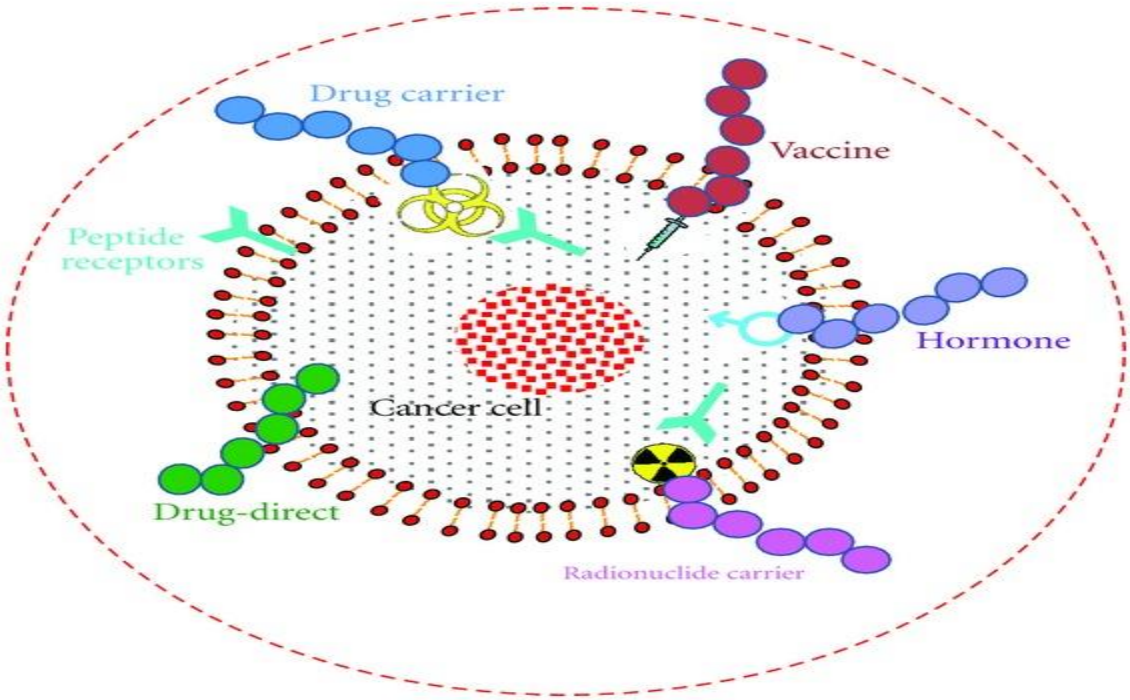


Fig. 1.3. Different possible treatment options of cancer using peptides. Peptides can be used as anticancer drug, cytotoxic drug carrier, vaccine, hormones, and radionuclide carrier.(Thundimadathil 2012)

The plasma membrane consists of a lipid bi-layer which is highly hydrophobic in nature and therefore hydrophilic compounds are unable to cross this membrane unless specific mechanism has evolved for this purpose. Some ions have the ability to cross through the channels that undergo regulated openings, whereas some hydrophilic molecules are carried through trans membrane shuttle proteins **(Joliot and Prochiantz 2004);** in the above two mentioned cases the cargo can directly access the cytoplasm. These mechanisms are not possible for larger protein and peptide molecules as these are internalized by endocytosis, resulting in uptake of cargo into endocytic vessels. Subsequent steps are needed for release into the cytoplasm.

Therefore, alternative approaches are thought that can enable a rapid and rational design of active and specific molecules. This design includes oligonucleotides, proteins and peptidomimetics **(Pavlou and Reichert 2004, Sillerud and Larson 2005, Aagaard and Rossi 2007)**. Due to poor bioavailability of these molecules, rapid and rational design is hampered. The success of these molecules strictly depends upon its delivery and targeting strategies **(Langer 2001).** In the outer leaflet of the plasma membrane, there are groups that are normally negatively charged; therefore the common strategy is the incorporation of the positive charge of various magnitudes which has the possibility of cellular uptake. Thus, cell-penetrating peptides (CPPs) are generally cationic in nature.

The peptides that have the ability to reach the cytoplasmic and/or nuclear compartments in the live cell are actually the CPPs. the mentioned ability was first found in the natural HIV Tat transactivator **(Frankel and Pabo 1988, Green and Loewenstein 1988)** and for the homeodomain Drosophila melanogaster transcription factor Antennapedia **(Joliot, Pernelle**

et al. 1991). Afterwards this research has been extended to non-natural peptides, some of which are found to be very efficient CPPs. Some of the CPPs peptides are summarized below in table 1.1:

Table 1.1. List and Sequences of cationic peptides including natural and non-natural peptides.

Peptide	Sequence	Class	Origin	reference
R9	RRRRRRRRR-NH2	Cationic CPP		(**Wender, Mitchell et al. 2000)**
Tat	C-(acetamidomethyl) GRKKRRQRRRPPQQ	Cationic CPP	HIV Tat protein (48-60)	**(Vivès, Brodin et al. 1997)**
Penetratin	RQIKIWFQNRRMKWKK	Cationic/amphipathic CPP	Antennapedia homeodomain	(**Derossi, Joliot et al. 1994)**
Buforin IIb	TRSSRAGLQWPVGRVHRLLRKd	Host defense peptide	Histone H2A (toad stomach)	**(Lee, Park et al. 2008)**
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL-NH2	Amphipathic CPP	Galanin and mastoparan	(**Pooga, Hällbrink et al. 1998)**
Vasostatin 1	MRSAAVLALLLCAGQVTALPVNSPMNKGDTEVMKCIVEVISDTLSKPSPMPVSQECFETLRGDERILSILRHQNLL-COOH	Bioactive CPP	Vasostatin(1-76)	**(Ramella, Boero et al. 2010)**
hLF	KCFQWQRNMRKVRGPPVSCIKR-NH_2	Cationic CPP	Human lactoferrin	**(Duchardt, Ruttekolk et al. 2009)**
(RXR)4	(R-Ahxa -R)4-PMO	Cationic CPP	Not Applicable	**(Abes, Moulton et al. 2006)**
TP10	AGYLLGKINLKALAALAKKIL-NH_2	Amphipathic CPP	Galanin and mastoparan	**(Soomets, Lindgren et al. 2000)**
Maurocalcine	GDCLPHLKLCKENKDCCSKKCKRRGTNIEKRCR-COOH	Bioactive CPP	Scorpion venom	**(Estève, Mabrouk et al. 2005)**
p14ARF	MVRRFLVTLRIRRACGPPRVRV-NH_2	Bioactive CPP	p14ARF protein (1-22)	**(Johansson, El-Andaloussi et al. 2008)**
M511	FLGKKFKKYFLQLLK-NH2	Bioactive CPP	Rodent angiotensin receptor	**(Östlund, Kilk et al. 2005)**

TBX2	GKMADWFRQTLLKKPKKRPNS PESTLQLRD ATPGGAIVS-NH2	Bioactive CPP	receptor 4 adaptor protein Toll- (19–43)	(**Low, Mortlock et al. 2007)**
Cyt c	GTKMIFVGIKKKEERADLIAYLK KA-NH_2	Bioactive CPP	Human cytochrome c (77-101)	**(Jones, Holm et al. 2010)**
mPrP	MANLGYWLLALFVTMWTDVG LCKKRPKP-NH2	Bioactive CPP	Mouse prion protein (1–28)	**(Löfgren, Wahlström et al. 2008)**
$SAHB_A$	EDIIRNIARHLAX[b]VGDX[b]N_L[c] DRSIW-NH_2	Chemically modified bioactive CPP	BID BH3	**(Walensky, Kung et al. 2004)**
Lactoferricin B]	FKCRRWQWRMKKLGAPSITCV RRAF[d] peptide	Host defense peptide	Bovine lactoferrin (17–41)	(**Mader, Salsman et al. 2005)**
The above-mentioned peptides have been used as CPP that have been attached with different peptides in different conditions.				

CPPs exhibits specific structural requirements based on the specific amino acid sequences in the regions of secondary structures such as amphipathic nature **(Eguchi, Akuta et al. 2001, Magzoub, Kilk et al. 2001, Christiaens, Symoens et al. 2002)** and alpha helical content that helps to provide clues regarding the mechanism of uptake. If substitution is done in any one of the amino acid sequences, then the cellular translocation can be inhibited **(Oehlke, Beyermann et al. 1997, Scheller, Wiesner et al. 2000)**. Considering Tat and Penetretin peptides, Tat is enriched with hydrophilic arginine residues; mutating any one of the arginine residues may abrogate its translocation activity. In penetretin W6 and F7 is crucial for its activity.

1.7 Pharmacokinetics and Pharmacodynamics of Peptides

Peptides are polypeptide chains containing at most 50 amino acids residues or less. Peptides exhibiting significant biological activity are either isolated from natural sources such as plants, animals and human or derived from chemical libraries or from genetic/recombinant

libraries. Bioactive peptides often serves as potent agonists or antagonists against various receptors involved in many disease progression **(Sato, Viswanathan et al. 2006)**. Some of the well-known peptides are gastrin releasing peptide in the treatment of cancer, Glucagon like peptide-1 (GLP-1) used in the control of diabetes and ghrelin to treat obesity.

1.8 Pharmacokinetic Characteristics of Therapeutic Peptides

In comparison to that of small organic molecule, peptides as drug or therapeutic agent is less successful. The main limitations which inhibit its use as a therapeutic agent **(Guichard 2004, Pichereau and Allary 2005)** are hepatic clearance, low oral bioavailability, high conformational flexibility, poor ability to cross physiological barriers because of their hydrophilicity, renal clearance, a short life because of their rapid degradation by proteolytic enzyme of the digestive system and blood plasma, internal risk of immunogenic effects and high synthetic and production cost **(Vlieghe, Lisowski et al. 2010)**. Depending on size, the cost of production may vary from 10-fold to nearly 100-fold more **(Bray 2003)**. In spite of the above-mentioned limitations, in some cases, peptides are considered first over small organic molecule as a therapeutic agent dur to e.g., higher affinity/specificity to target and lower toxicity profiles. A close comparative study of small molecule and peptide as a therapeutic agent is furnished in the table 1.2.

Table 1.2. Comparison Between Peptides and Small Molecules Small Molecules Peptides

Characteristic	•Small molecule	•peptides
Availability	•More than 80% of drugs in market	•Less than 2% of drugs in market
Bioavailability	•Good oral bioavailability	•Poor oral bioavailability
Permeability	•higher permeability	•Low permeability
Stability	•stable	•Less stable
Cost	•Low cost	•High cost
Half life	•Many of it have high half life	•Short half life
		• Excellent target specificity • Low toxicity and immunogenicity • Mechanism of action well understood • High binding affinity • Easier to identify targets • Broad disease targets • Low risk of drug–drug interaction potential • Lower impact from generics

1.9. Chemical strategies to improve the pharmacokinetics of peptides

1.9.1. Proteolysis of peptides

Peptides contain amide bonds and therefore undergo proteolytic degradation susceptibly by enzymes such as peptidases and proteoses. In addition to these two mentioned enzymes, other two classes of enzymes such as membrane-bound enzymes including carboxypeptidase, aminopeptidase, and endopeptidases, and luminally secreted enzymes such as chymotrypsin, trypsin, pepsin, and elastase plays a crucial role proteolytic degradation of peptides. The most vital route of eliminating peptides through the body is proteolytic degradation and urinal clearance of peptides that may exceed cardiac output due to blood degradation.

N-terminal residues in the peptides are highly important as the half-life of peptides in plasma is generally related to it. Normally it is observed that peptides containing Threonine, Serine, Methionine, Alanine, Glycine, or Valine in its N- terminus residues have a longer half-life in comparison to the peptides containing Lysine, Arginine, Phenylalanine, Leucine and Aspartic acid in its N-terminal residues. The most extensively peptide enzymatic degradation occurs in that peptide whose domain is rich in Proline, Threonine, Serine, and Glutamine residues. Proteolytic enzymes, their substrates, and site specificities are well established **(Woodley 1994, Werle and Bernkop-Schnürch 2006)**. Moreover, a number of software programs are available which helps to predict the cleavage site of various peptides. Once the peptide cleavage site is known, then a well-directed modification of the peptide can be adopted in order to minimize the proteolytic degradation (**Werle and Bernkop-Schnürch 2006**).

1.9.2. Stabilization of peptides inhibiting proteolysis

The stability of peptides can be enhanced against proteolysis. Many approaches are available for this purpose **(Woodley 1994, Werle and Bernkop-Schnürch 2006)**. Some of the approaches are hereunder through which renal clearance can also be inhibited

1) Cyclisation of the peptides
2) modification of N-or/and C-termini of the peptides.
3) Replacing L-amino acid by D-amino acid
4) conjugation of the peptides with macromolecules

Cyclisation which not only increases the stability but also the permeability of the peptides. It introduces conformational strain inhibiting flexibility of peptides. Peptides can be cyclized head to tail, head/tail to side chain or side chain to side chain depending upon the functional group present in the molecule. Lactamization, lactonization and sulphide based bridges are the common route for the cyclization of peptides **(Goodwin, Simerska et al. 2012)**. Cyclic enkephalin analog was found to be highly resistant to enzymatic degradation **(Weber, Greene et al. 1992)**. A cyclic peptide derived from herpes simplex virus is found to be stable in 50% human serum but its linear is completely unstable **(Angelini, Morales-Sanfrutos et al. 2012)**.

Another important structural modification of peptide includes the modification of N-or/and C-termini that can also enhance peptide stability for these peptides which are susceptible to exopeptidase-mediated proteolysis. The two common ways of such modification is the N-acetylation and C-amidation **(Ferdinandi, Brazeau et al. 2007)**. For example, native somatostatin is found to be less stable in comparison to that of the modified i.e., N-acetylated

somatostatin **(Wender, Mitchell et al. 2000)** similar is the case with GLP-17-34 used in diabetes N-acetylated form is found to be more stable than that of unprotected form **(Chen, Gfeller et al. 2013)**. Tesamorelin approved by FDA in 2010 for the reduction of excess abdominal fat in HIV infected patients with lipodystrophies, is one of the such example. Tesamorelin consists of a synthetic 44 amino acid sequence of human growth hormone releasing hormone (GHRH) with a hexenoyl moiety attached to the tyrosine residue at the N-terminus. Tesamorelin was found to be resistant to dipeptidyl peptidase-4 degradation and its half-life in healthy human subjects is much longer than that of natural GHR**H (Werle and Bernkop-Schnürch 2006, Ferdinandi, Brazeau et al. 2007)**.

In order to increase the stability of therapeutic peptides the most widely used method is the substitution of the most widely used metabolically labile amino acid i.e., the peptide stability can also be increased by substituting natural L-amino acid by non-natural D-amino acid as it decreases the substrate recognition of the proteolytic enzyme. In this regard, a number of examples were reported with some exceptions. The examples are the replacement of specific glycine with D-serine in the bicyclic peptide the inhibitor of cancer-related protease urokinase plasminogen activator not only improves potency by 1.8 times but also increases the stability by 4 fold in mouse plasma **(Chen, Gfeller et al. 2013)**, L-arginine containing vasopressin has a half-life of 10-35 minutes in humans whereas D-Arg containing analog desmopressin has a half-life of 3.7 hours in a healthy human. The exception in this regard is dermorphin analog with additional D-amino acid substitution more rapidly cleaved in comparison to that parent peptide as it exhibits remote secondary structural features which are essential for enzyme recognition.

The first selective growth hormone releasing peptide is the pentapeptide Ipamorelin. It is a pentapeptide with the amino acid sequence Aib-His-D-2-Nal-D-Phe-Lys-NH_2 that was derived from GHRP-1 **(Raun, Hansen et al. 1998)**. The resulting terminal half-life for ipamorelin is approximately 2h **(Gobburu, Agersø et al. 1999)**.

Renal clearance is assumed to be another major cause that inhibits the development of peptides as a successful therapeutic agent. The hydrophilic peptide normally has a molecular weight ‹ 2-25KDa and is easily reabsorbed by the renal tubule and therefore susceptible to frequent filtration through the glomeruli of the kidney having a pore size of ~ 8nm. Endocytosis and degradation by the proteasome and the liver are the other minor route of peptide clearance. Comparative studies of systemic and renal clearance in animal models have explained that renal clearance is the major elimination pathway.

1.9.3. Strategies to inhibit renal clearance

Two common strategies that can inhibit renal clearance, as well as proteasomal degradation, of a peptide, are the

a) Binding of the peptide to a membrane protein or a serum protein.

b) Conjugation of the peptide to large polymers.

Glomerular filtration can be reduced by covalently linking albumin-binding small molecules to peptides. Indirect interaction of the peptide with albumin through the highly bound small molecule improves proteolytic stability and prolongs the half-life **(Pollaro and Heinis 2010)**. For example, when an albumin binding peptide was linked to a bicyclic peptide, it became resistant to proteolysis and 50-fold increase in half-life **(Raun, Hansen et al. 1998)**.

Renal clearance of peptide is also reduced when the molecular weight and the hydrodynamic volume of the peptide are increased. This can be done by the conjugation of peptides to a large synthetic molecule or carbohydrates or natural polymers. The most commonly used polymers for this purpose are polyethylene glycols (PEG), hydroxyethyl starch (HES), and polysialic acid (PSA).

1.10. Pharmacokinetics of therapeutic peptides

1.10.1. Immunogenicity

The tendency of a therapeutic agent to bring out an unwanted immune response is referred to as immunogenicity. It is an important concern for protein therapeutics and often results in the formation of anti-drug antibodies (ADA) after repeated and chronic administration **(Ferdinandi, Brazeau et al. 2007)**. ADA formation may not only regulate or abrogate the biological activity of the therapeutic agent but may also modify its pharmacokinetic profile.

Small peptides of molecular weight less than 4KDa are generally believed to be poor immunogens. However, due to the complexity of the human immune response, few exceptions have been observed **(Rosenstock, Balas et al. 2013)**. One of the most recent examples is taspoglutide, the first one weekly GLP-1 analogue based on a human sequence. As a result of relatively uncommon but serious hypersensitivity reaction that could be related to ADA formation, its development was discontinued **(Rosenstock, Balas et al. 2013)**.

The administration pathway may affect immunogenicity through the route of administration cannot render a peptide or a protein immunogenic, it can increase the chance of

an immune reaction to a therapeutic that already has immunogenic properties **(Schellekens 2005)**. The higher incidence of immunogenic reaction is associated with the subcutaneous route of administration in comparison to that of the intramuscular or intravenous route. This is because in the subcutaneous route of administration the increased likelihood of the formation of protein aggregates which are a factor known to increase immunogenicity **(Egrie and Browne 2001)**.

The immunogenicity of peptides can be avoided by avoiding antigenic sequences in the amino acid sequence and structural modification such as glycosylation or PEGylation as these can shield antigenic determinants on the drug through steric hindrance from detection by the immune system **(Ferdinandi, Brazeau et al. 2007)**. For example immunogenicity of wasp venom a 17 residue peptide (Vespula kinin, VSK-1) was dramatically when carbohydrate was attached to it**(Sato, Viswanathan et al. 2006)**. Another example is PEGylated TPO mimetic peptide caused no immune mediated lesions in mice, but recombinant human TPO suppressed mega karyocytopoiesis and caused B-lymphocyte hyperplasia in lymphoid tissue in mice, consistent with antigenic stimulation **(Camenisch, Alsenz et al. 1998)**. In order to understand why a peptide sequence is non-immunogenic or immunogenic requires more research.

1.11. Nanoscale Delivery of peptides

Peptides are highly vulnerable molecules in spite of their incredible selectivity and their ability to provide effective and potent action because of their short in vivo half-lives and pre-systematic degradation by enzymes either at the site of administration or in every anatomical location on their way to the site of pharmacological action. This factor restricts its use in oral

delivery which is the most common method for drug administration with a high level of patient acceptance. Apart from the above-mentioned poor membrane stability and high molecular weight of peptides have remained unsolved issues. Among the diverse strategies, nanotechnology has provided a glimpse of hope in the oral delivery of peptide drugs. New biomedical applications have been created due to the small size, customized surface, improved solubility, and multifunctionality of nanoparticles.

Due to their hydrophilic nature, most of the therapeutic peptides would not be expected to follow the trans cellular route of absorption through passive diffusion **(Camenisch, Alsenz et al. 1998)**. The macromolecules cannot be absorbed by paracellular route as the dimension of paracellular route lie in between 30-50^{0}A and therefore it is restricted to relatively small hydrophilic molecules that can fit in these spaces **(Rubas, Cromwell et al. 1996)**. In order to provide high transfer of drug across the epithelium mucosa, biologicals in most of the oral strategies used a system equipped to protect against enzymatic degradation even in the harsh environment of the GI tract. In Peyer's patches, epithelial cells or lymphoid tissues takes up certain particles, one of them being nanoparticles **(Morishita and Peppas 2006**). M cells are specialized epithelium covers the follicle of lymphoid tissue in the Peyer's patches **(Brayden, Jepson et al. 2005)**. M cells are responsible for particle uptake and surface charge and size of particles are the important factor governing the uptake of particulate by the m cell **(Shakweh, Besnard et al. 2005)**. Generally, the transport of particles across the mucosal epithelium is favored by the nanoscale dimension. Desai et al.,**(Desai, Seekatz et al. 2016)** demonstrated that 100 nm poly (lactic-co-glycolic acid) diffused through the submucosal layer whereas 10 µm particles were predominantly localized on the epithelium lining of the tissues. For the

development oral delivery system for macromolecules, the nanoscale carriers composed of biocompatible polymers are thought to be promising. For oral peptide administration, the potential of Chitosan has been recently reported by several researchers. When diabetic rats were administered orally with insulin-loaded chitosan nanoparticles, their glucose levels were reduced to normal range for several hours **(Pan, Li et al. 2002, Ma, Lim et al. 2005)**. However, chitosan-coated nanoparticles clearly reduced the transepithelial resistance of a Caco-2 cell monolayer **(Garcia-Fuentes, Prego et al. 2005, Prego, García et al. 2005**). Therefore, their potential use for clinical purposes is questionable.

In recent years, research in this field gravitated towards use of polymeric micelles as functional nanomaterials **(Aliabadi and Lavasanifar 2006)**. In an aqueous environment these are formed by the self-assembly of amphiphilic block copolymers. The hydrophobic core in the nanoscopic core/shell structure of polymeric micelles acts as a micro reservoir for the encapsulation of hydrophobic drugs. Among them biomolecules such as DNA and enzymes are trapped by poly-ion complex micelles and this micellar structure helps them to attain increased stability against various environmental factors **(Yuan, Harada et al. 2005)**. Recently it has been shown that the polymeric micelles cross the intestinal barrier **(Mathot, van Beijsterveldt et al. 2006)**, therefore it might also be useful for the oral delivery of peptides.

1.12. S100 Proteins

The S100 proteins are a family of proteins that contains 20 known human members each coded by a separate gene belonging to calmodulin/parvalbumin/troponin C family. These are Ca^{2+} modulated proteins. Out of 20 members, 9 of these are identified i.e., from S100A1 to

S100A9 from the long arm of the human chromosomes 1(i.e., 1q21) **(Schäfer, Wicki et al. 1995)**. These proteins, normally found in vertebrates, are proteins of low molecular weight 10-12KDa and are of acidic in nature **(Salama, Malone et al. 2008)**. The first member of this protein family was introduced by Moore in 1965 who used for the first time a subcellular fraction from human bovine serum. It was named because of its solubility in 100% ammonium sulphate solution **(Moore 1965, Zimmer, Cornwall et al. 1995)**.

1.13. Structure of S100 Proteins

Ca^{+2}- binding EF-hand motif is conserved among the members of S100 proteins which induces conformational changes in the S100 proteins, exposing more hydrophobic areas, and this may facilitate the interaction of S100 proteins with secondary effector molecules **(Harpio and Einarsson 2004)**. These proteins have the capacity to form homodimers, heterodimers, and oligomers, whereas the majority of the members exist as a homodimer preferably. Only a single protein of the S100 family i.e., calbindin 3 occurs as a monomer. The Ca binding motif in these proteins is normally an EF-hand motif which is composed of two helices E and F joined by a loop that contains Ca binding regions. There are two distinct EF-hands in S100 proteins, one, common to all EF-hand motifs, on the C-terminal portion which contains the classical calcium-binding motif having a typical sequence of 12 amino acids and is flanked by the helices HIII and HIV. The other specific to this family is situated at the N-terminus **(Donato 1986, Heizmann, Fritz et al. 2002)** which is the characteristics of S100 protein. This EF-hand, with 14 consensus amino acids is flanked by helices HI and HII. It is S100 specific or called pseudo-EF-hand. Between the two EF-hand proteins, there is a region called “hinge”. After the C-terminal EF-hand region, there is a stretch of amino acids referred to as the C-terminal extension. It is the C-

terminal extension and the hinge area which creates variability among the different members of proteins.

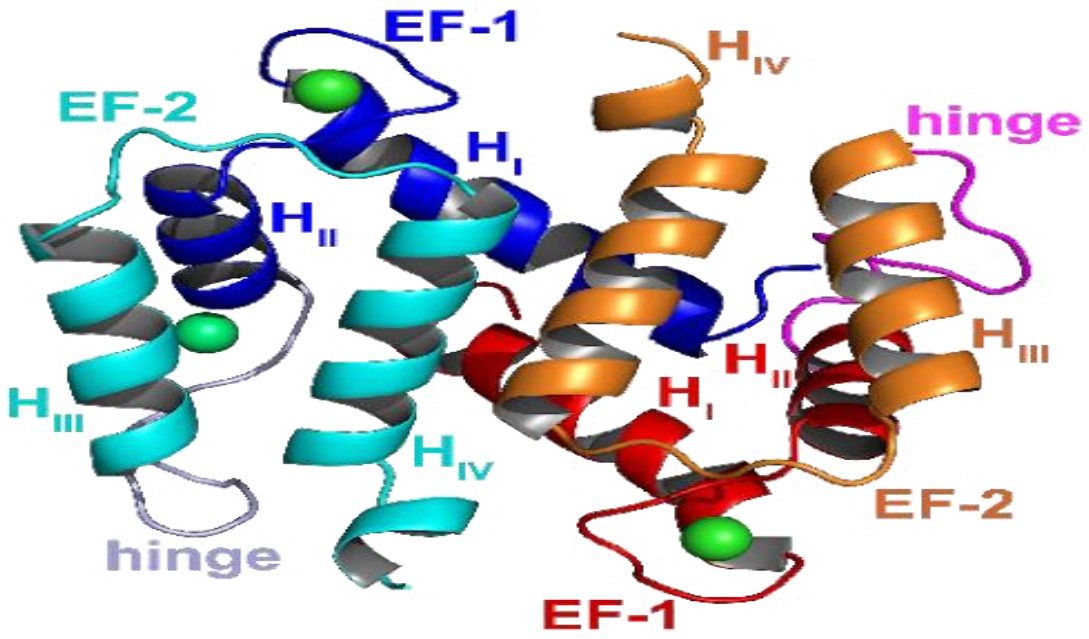


Fig: 1.4: Structure of S100 proteins (ref: Atlas of Genetics and cytogenetics in Oncology and hematology).

1.14. Functions of S100 proteins

A wide range of the intracellular and extracellular functions of this family of proteins is well established in the literature. A wide range of intracellular functions includes improvement in calcium homeostasis, regulation of transcriptional factors, enzymatic activity, signal transduction via protein phosphorylation, and regulation of cytoskeletal components. Again, they display a remarkable diversity of functions exhibiting direct interaction with tubulins, intermediate filaments, actin, myosin, and tropomyosin **(Santamaria-Kisiel, Rintala-Dempsey et al. 2006)**. A number of members, such S100A1 and S100A11, exhibit a vital role in

modulating proliferation; they are found to be potent in inhibiting cell proliferation **(Donato 2001)**.

Some of the members of this family act as leukocyte chemoattractant. macrophage activators, and modulator of cell proliferation specifically when they are present as extracellular proteins **(Cornish, Devery et al. 1996, Yen, Harrison et al. 1997, Hiratsuka, Watanabe et al. 2006, Low, Mortlock et al. 2007)**. The variety of pathologies, such as inflammation and cardiomyopathies and their roles in carcinogenesis, begin to explain intracellular and extracellular functions associated with S100 proteins. Some S100 proteins act as tumor promotors whereas others act as tumor suppressors. Other functions in which S100 proteins are thought to be involved include cell differentiation, cell motility, transcription, and cell cycle progression.

1.15. Function of the S100B protein

S100B has several properties. Intracellularly it is a normal part of calcium hemostasis, thereby transferring the signal from the second messenger. It is also involved in cell differentiation and cell cycle progression, and it has been shown to inhibit apoptosis **(Brewton, Haddad et al. 2001)**. Extracellularly, in both normal physiology and during traumatic conditions, administered S100B promotes neurogenesis and normal plasticity **(McAdory, Van Eldik et al. 1998, Nishiyama, Knopfel et al. 2002)**, performs neuromodulating actions and enhances processes involved in memory and learning.

However, the effect and physiological functions of S100B have been shown to be concentration-dependent, where lower concentrations (nanomolar level) are beneficial and

higher concentration levels (micromolar level) are correlated with harmful effects **(Rothermundt, Peters et al. 2003, Van Eldik and Wainwright 2003)**. High extracellular levels of S100B resulted in neuronal malfunction or cell death **(Hinkle, Baldwin et al. 1997, Hu, Ferreira et al. 1997)**. The different effects of S100B have been suggested to depend on the receptor for advanced glycation end-products (RAGE), which is upregulated by S100B levels and may cause proinflammatory gene activation **(Donato, Cannon et al. 2013)**, although how S100B exerts its biochemical actions is still unclear.

1.16. p53 and its interaction with S100B

In the field of cellular biology, p53 has gained immense attention due to its involvement in many disease etiologies. The gene is known as TP53 which codes for a protein that regulates the cell cycle and therefore functions as a tumor suppressor. It is very important for cells in multicellular organisms to suppress cancer. P53 has been described as the "Guardian of the genome" referring to its role in conserving genome stability. This name is also due to its molecular mass; it is in the 53 kDa fraction of cell protein.

p53 plays a remarkable role in preventing tumor development. A range of oncogenic stresses results in p53-mediated cell cycle arrest and apoptosis, thus successfully inhibiting tumor progression. More than 50% of adult human cancers have mutation and deletion in TP53 genes. Thereby its importance as tumor-suppressing activity is highlighted. The p53 pathway may be altered by other oncogenic events in many cancers where p53 is present in the wild-type form. Thus, in most cancers, the p53 response may be defective. The human wild-type p53 protein consisting of 393 amino acid residues comprises several domains:

- Trans activation domain (TAD) containing 1-44 at the amino acid terminus part is responsible for activating downstream target gene.
- A proline rich domain containing 58-101 amino acid residues and it responds to DNA damage through apoptosis.
- The amino acid residues 102-292 comprise the DNA binding domain (DBD) which is a core domain consisting of a variety of structural motifs. Most mutations even more than 90% mutations occur in this domain. In human cancers, a single mutation in this domain can cause a major conformational change.
- The oligomerization domain (325-356) consists of a β strand that interacts with another p53 monomer to form a dimer, which is followed by an α helix that mediates the dimerization of the two p53 dimers to form a tetramer.
- Three presumed nuclear localization signals (NLS) have been identified in the C terminus through sequence similarity and mutagenesis. The most N terminal NLS which consists of three consecutive lysine residues to a basic core is the most active and conserved domain.
- Two recognized nuclear export signals (NES) have also been identified. The leucine-rich C terminal NES found within the oligomerization domain, is highly conserved and it has been suggested that oligomerization can result in masking of the NES, resulting in

p53nuclear retention.

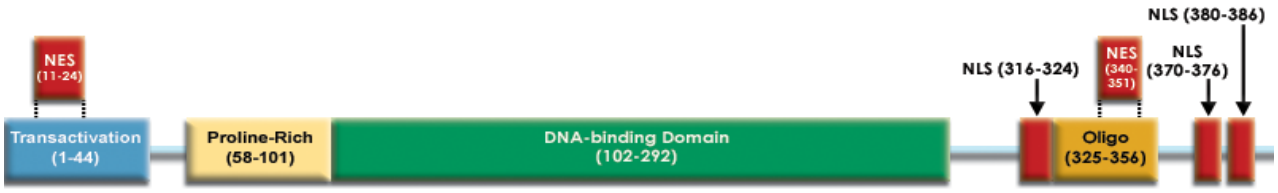


Figure 1.5: Amino acid sequence of the p53 protein.

1.17. Relation of S100 proteins with Cancer

Human ailments such as cancer, diseases of the skin and cardiovascular diseases are due to dysregulation of S100 gene expression. In patients with malignant melanoma at different stages, an increased intertumoral level of S100B has been detected specifically in stage IV and to some lesser extent has been detected in thyroid carcinoma and renal cell carcinoma **(Molina, Navarro et al. 2002)**. The degree of malignancy and the Strength of S100B expression were found to be directly correlated with one another. It is interesting to note that there was also an inverse relationship between S100B expression and duration of survival. In vitro, S100B inhibits calcium-dependent phosphorylation of p53 by protein kinase C. This results in the suppression of the p53 tumor suppressor mechanism which is of great importance in malignant melanoma as well as in other tumors. Consequently, uncontrolled growth in tumors is observed **(Donato 1991, Lau, Devery et al. 1995, Zimmer, Cornwall et al. 1995, Molina, Navarro et al. 2002)**. Thus, the increased expression level of serum S100B also correlates with reduced survival and has been shown to reflect tumor load, stage, and prognosis. And is an independent poor prognostic factor in melanoma. This allows S100B to be used as a diagnostic marker and for the

staging of malignant melanoma in a clinical setting. S100B also proved to be a valuable marker in assessing a patient's response to treatment **(Hamberg, Korse et al. 2003)**. A good response to therapy, as well as treatment, is observed by decreased level of S100B I.e., related to an increased level of survival. Furthermore, has a chance of reoccurrence when an elevated level of serum S100B is noted following treatment.

1.18. Fluorescence Anisotropy

Measurements of fluorescence anisotropy are a powerful tool in structural biology and related areas. Upon excitation from polarized light, the emissions from many samples are also polarized. Anisotropy is the term that is used to determine the extent of polarization of the emission. Samples that are supposed to display polarized emissions have nonzero anisotropies. The origin of anisotropy is the existence of transition moments for absorptions and emissions that lie along a specific direction within the fluorophore structure. In a homogeneous solution, the ground state fluorophore is all normally oriented. When exposed to polarized light, those fluorophores that have their absorption transition moments oriented along the electronic vector of the incident light are preferentially excited, hence the excited state population is partially oriented along the electronic vector of the polarized exciting light.

Polarization of fluorescence occurs as a result of many processes. All chromophores have transition moments along a specific direction. One of the common causes of depolarization is the rotational diffusion that changes the direction of the transition moments. Solvent viscosity, size and shape determine the rotational diffusion rate. The rotational rate of

fluorophore in solution is dependent upon the viscous drag imposed by the solvents. The change in fluorescence anisotropy results due to the change in the solvent viscosity. For small fluorophores in low-viscosity solution, the rate of rotational diffusion is typically faster than the rate of emission under that condition the emission is depolarized and the anisotropy is close to zero.

A polarized light striking a fluorescent molecule result in polarized fluorescence. Depending on rotational diffusion and other factors, the polarized emission gradually returns to unpolarized fluorescence. Anisotropy is directly related to polarization and which is the ratio of the polarized light component to the total light intensity. With an optional polarizer installed in a spectrofluorometer, light intensity is defined as IVV with excitation and emission polarizer

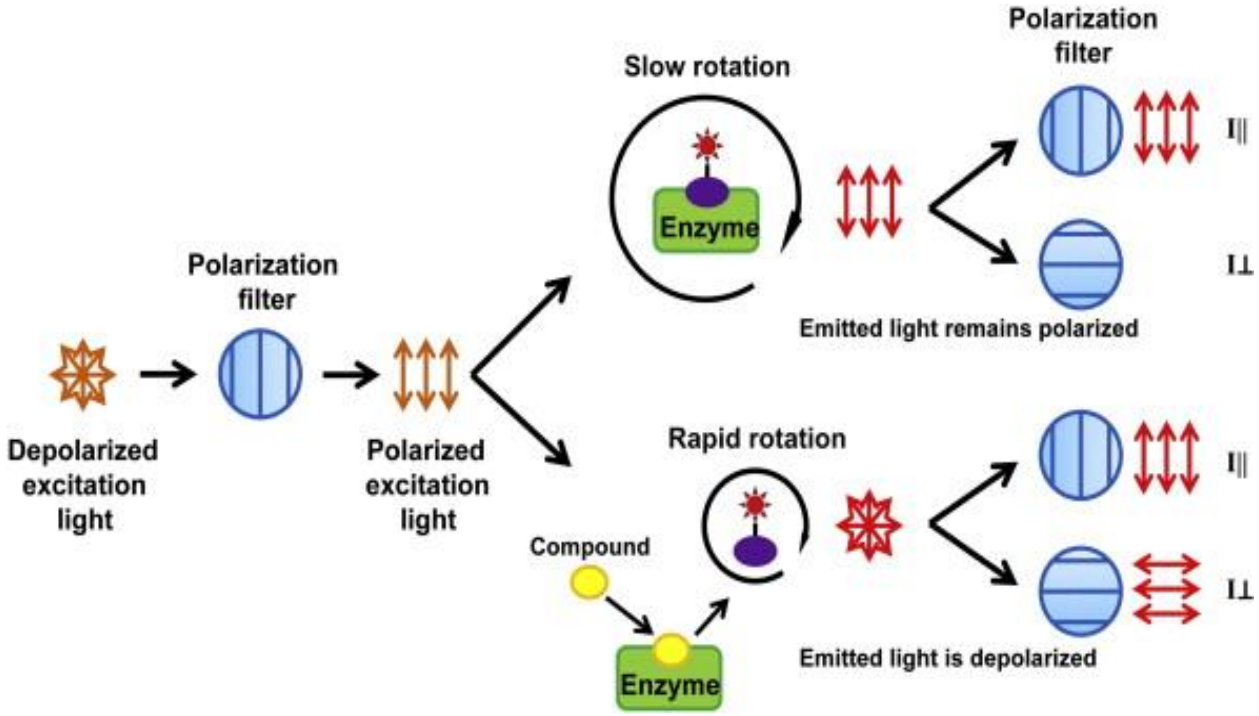


Figure 1.6: Fluorescence polarisation Ref:(Shishkova, Zeng et al. 2017)).

mounted vertically; IHH is for excitation and emission polarizer mounted horizontally IHV uses

an excitation polarizer horizontally and the emission polarizer vertical IHV requires the excitation

polarizer vertically and emission polarizer horizontally. The basic set of L-format is shown in figure 7.

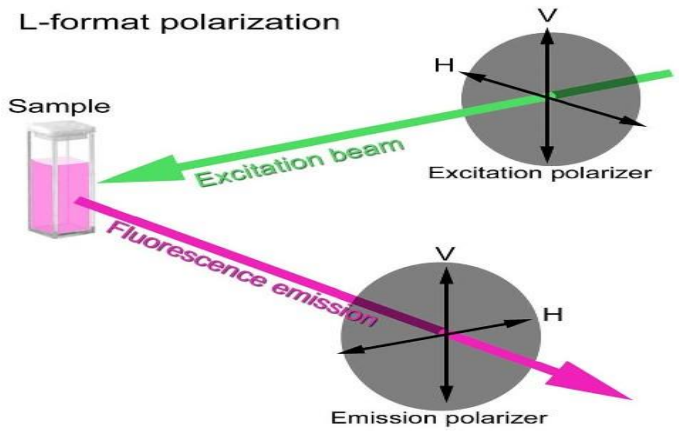


Figure 1.7: Schematic representation of L format polarization (HORIBA)

Anisotropy < r > is defined as follows

$$\langle r \rangle = \frac{I_{VV} - G * I_{VH}}{I_{VV} + 2 * G * I_{VH}}$$

Where G the G factor is as follows.

$$G = \frac{I_{HV}}{I_{HH}}$$

1.19. Thalassemia

Among the most common monogenic diseases (that are caused by variations in a single gene and are typically recognized by striking familial inheritance patterns) sickle cell anemia and its variants and thalassemia are highly well known and most spreading among people. It is an inherited disorder of hemoglobin (Hb) from the parents **(Weatherall and Clegg 2008, Williams and Weatherall 2012)**. Thalassemias are the most common genetic disorder worldwide occurring more frequently in the Mediterranean **(Fawdry 1944)**, the Indian subcontinent, Southeast Asia and West Africa **(Weatherall, Edwards et al. 1968)**.The main causes of anemia are ineffective bone marrow erythropoiesis and excessive red blood cell hemolysis **(Marengo-Rowe 2007)**.

Mature erythrocytes contain essentially equimolecular amounts of alpha and beta chains which are manufactured by reticulocytes also in equimolar amounts. Patients suffering from thalassemia do not produce enough hemoglobin (Hb) A ($\alpha 2\beta 2$) because of their inability to manufacture either the functional alpha or beta polypeptide chain of human hemoglobin. The production of the alpha chains is only depressed in alpha thalassemia, and beta-thalassemia depresses only the production of the beta chains. Both alpha- and beta-thalassemia may occur in the major (homozygous), intermediate, and minor (heterozygous) genetic forms clinically and also can interact with the presence of abnormal hemoglobin in the same individual. It is necessary to outline the interplay of the various polypeptide chains of hemoglobin during normal human development in order to explain the nature of thalassemia syndrome. In the first trimester of intrauterine life, zeta, epsilon, alpha, and gamma chains attain significant levels and in various combinations form Hb Gower I ($\zeta 2\varepsilon 2$), Hb Gower II ($\alpha 2\varepsilon 2$), Hb Portland ($\zeta 2\gamma 2$),

and fetal hemoglobin (HbF) (α2γ2) **(Schroeder, Huisman et al. 1968)**. HbF is produced from approximately eight weeks of gestation through birth and constitutes 80% of Hb in the full-term neonate. During the first few months of life and, in the normal state, constitutes <1 percent of total Hb by early childhood. Hb F is composed of two alpha globins and two gamma globins (α2γ2). Whereas Hb Gower and Hb Portland soon disappear, HbF persists and forms the predominant respiratory pigment during intrauterine life. Before birth, gamma-chain production begins to wane so that after the age of 6 months postpartum, only small amounts of HbF (<2%) can be detected in the blood **(Marengo-Rowe 2007)**. In early intrauterine life, beta-chain synthesis is maintained at a low level but gradually increases to significant concentrations by the end of the third trimester and continues into neonatal and adult life. The synthesis of delta chains remains at a low level throughout adult life (<3%). Hence during normal development, the synthesis of the embryonic hemoglobin's Gower and Portland is succeeded by the synthesis of HbF, which in turn is replaced by the adult hemoglobin's, HbA and HbA2. HbA2 is a minor adult Hb that normally accounts for approximately 2.5-3.5% of total Hb from six months of age and onwards.

CHAPTER 2

SYNTHESIS AND CHARACTERIZATION OF A BRANCHED BIDENTATE PEPTIDE

Protein-protein interactions are a vital component of cells. Inhibition of dysregulated protein-protein interactions is essential for developing drugs for many diseases. Although a few small molecule protein-protein interaction inhibitors have been developed, in general, It is difficult to develop small molecule Inhibitors of protein-protein interactions. This is due to the very nature of the protein-protein interfaces which are usually shallow and hydrophilic. Since the protein-protein interfaces consist of peptides, constrained peptides are easier to develop against protein-protein interfaces. However, they tend to have lower affinities than desired for in vivo studies. In this study, we have attempted to develop a protein-protein interaction inhibitor by dimerizing a peptide that binds reasonably well to a dimeric protein by dimerizing the peptide itself with a designed spacer.

We have chosen S100B as the target protein. S100B is a dimer and a peptide, named TRTK, has been reported that tightly binds to the S100 B as a monomer. However, affinity may not be sufficient to inhibit S100B within the cell. In order to develop a tight binding inhibitor, we have chosen to constrain this peptide by introducing alpha-amino isobutyric acid at the opposite face through which the peptide interacts with S100B. We have linked two such peptides through a designed linker to generate a dimeric molecule that binds the target at a significantly higher affinity and attempted to find out the pharmacokinetics and pharmacodynamics of such a molecule within a live animal system.

2.1. Materials

2.1.1. Chemicals and Reagents

Fmoc-amino acids, Rink amide MBHA resin, 2-(1H-benzotriazole-1-yl)-1, 1, 3, 3-tetramethylaminiumtetrafluoroborate (TBTU), hydroxybenzotriazole (HOBt) and N, N, N', N'-tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU) were purchased from Novabiochem. N, N, -diisopropylethylamine (DIPEA), thioanisole, 1, 2-ethanedithiol (EDT), trifluoroacetic acid (TFA), ammonium iodide (NH4I), dimethyl sulfide (Me_2S) and HPLC grade acetonitrile were purchased from E-Merck, Germany. Triisopropylsilane (TIS) was purchased from Sigma Chemical Company ***(Egerstedt and Mishra 2008)***. N, N-dimethylformamide (DMF), diethyl ether (Et2O), piperidine and HPLC grade water were procured from Spectrochem. Reverse-phase Hypersil Gold C-18 HPLC column was acquired from Thermo Corporation Limited. Cuvettes for spectroscopic analysis were purchased from Hellma Analytics. NMR spectroscopy tubes (Wilmad) were procured from Sigma-Aldrich.

2.1.2 Peptide synthesis and purification

All peptides were synthesized based on 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with a capping step consisting of 5% acetic anhydride and 5% lutidine in DMF after each coupling on Rink Amide PEGA resin using a peptide synthesizer (Protein Technologies International). Fmoc-amino acids were activated with TBTU in the presence of HOBt and DIPEA. Peptides were cleaved from the resin and side-chain protecting groups were removed by incubating with 94% TFA, 2.5% EDT, 1.5% Thioanisole, 1.5% water, and 0.5% TIS for 3 hours at 25 °C. In the case of methionine-containing peptides, reducing agents NH4I and Me2S were added to the cleavage cocktail mentioned above in order to prevent the oxidation of methionine. The crude peptides were purified by HPLC on a reverse phase Hypersil Gold C52 and C18 column using 0.085% Acetonitrile in 0.1% TFA and characterized by Electron spray

ionization mass spectrometry (ESI-MS) and Matrix assisted laser desorption ionization-Time of flight (MALDI-TOF).

2.1.3. Peptide labeling

The peptides were labeled with 5(6)-carboxyfluorescein in the solid phase. Dry resin-bound N-terminal deprotected peptide (3µmole) was taken in a 2 ml polypropylene syringe and reacted with a 10time molar excess of 5(6)-carboxyfluorescein, HBTU (1:1) and a 20-time molar excess of DIPEA in DMF. The reaction was kept for 4 hours in dark at 25 °C. Masses and purity of the labelled peptides were checked by ESI-MS and MALDI. After completion of the reaction, the resin was washed thoroughly with 20% piperidine in DMF until the wash solution becomes colorless. The resin was then washed consecutively with DMF and Et2O five times and finally dried under N2 atmosphere. After labeling, peptides were cleaved as unlabeled peptides and purified by HPLC, and masses were checked by ESI-MS and MALDI-TOF.

2.1.4 Fluorescence spectroscopy

Fluorescence was measured using a Quanta master 6 (PTI) T-geometry and Perkin Elmer L-geometry fluorometers. The fluorescence experiments were carried out at 25 ºC. The experiments were carried out in 1 cm pathlength cuvettes. The bandpasses for excitation and emission were 5 nm unless mentioned otherwise. Values were corrected for buffer fluorescence value, volume, and inner filter effect. The data were fitted into a single site binding equation considering 1:1 binding stoichiometry and dissociation constant (K_d) values were obtained by fitting them in Ky Plot according to the equation given below:

$$Y=A3+(A1-A3)\ *((A2+X+A4)-SQRT((A2+X+A4)\ *(A2+X+A4)-4*X*A4)/(2*A4)$$

where A1 is the final limiting value, A2 is the dissociation constant (K_d), A3 is the initial value, A4 is the fluorescent labeled ligand concentration and X is the titrant concentration.

2.1.5 CD spectroscopy

Circular Dichroism (CD) spectra were measured in a JASCO spectropolarimeter. The measurements were conducted in 50 mM Tris-HCl, pH 8.0 at 25 °C. The measurements were done with a bandwidth of 1.0 nm and a scan speed of 50 nm/min; a 1 mm pathlength cuvette was used throughout. Ten spectra were averaged to improve the signal-to-noise ratio. The sample concentrations were 5 μM. Secondary structure contents were estimated by a built-in secondary structure algorithm within the Spectropolarimeter. The algorithm uses the reference CD spectra of Professor Jen Tsai Yang, University of California, as the basis for the estimation. The program executes secondary structure estimation and calculates structural components including α-helix, β-sheet, turn, and random coil. Information includes the overlaid CD spectrum of protein/peptide, the CD spectrum of protein/peptide being calculated, and the mean residue CD spectrum.

2.2. Result and Discussions

In order to design a peptidomimetic that can function as a peptide-based inhibitor of S100B-p53 interaction, a conformationally constrained helix of 12-mer TRTK derived from Cap Z protein was designed in our laboratory. Cap Z protein tightly binds S100B protein encompassing the region of (TRTKIDWNKILS) TRTK-12. Since the binding site of p53 and Cap Z on S100B protein overlaps partially and encompasses the TRTK-12 region was conceived that a peptide containing the TRTK-12 sequence will compete with p53 for S100B. It is evident from the

structure of S100B and TRTK-12 complex that the C-terminal half of the bound peptide is helical, while the N-terminal part is an extended conformation.**(Garbuglia, Verzini et al. 1999)**

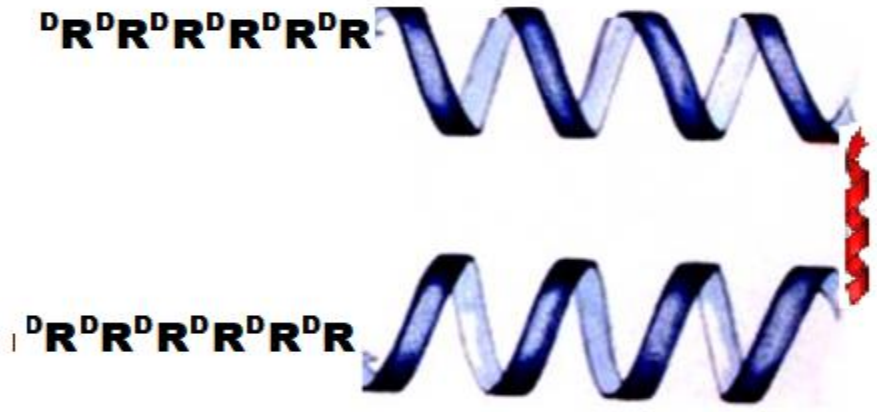


Figure 2.2: Schematic representation of the structure of the branched bidentate TRTK peptide with Aib substitution(^{D}R-means D-Arginine).

In the bound peptide, particularly at the helical section, N8 and S12 which are nonessential residues as these are not involved in the interaction have been substituted with Aib (aminoisobutyric acid) in the TRTK-12 sequence to derive the Aib substituted TRTK-12 peptide (TRTKIDWBKILB) (Figure 2.3).

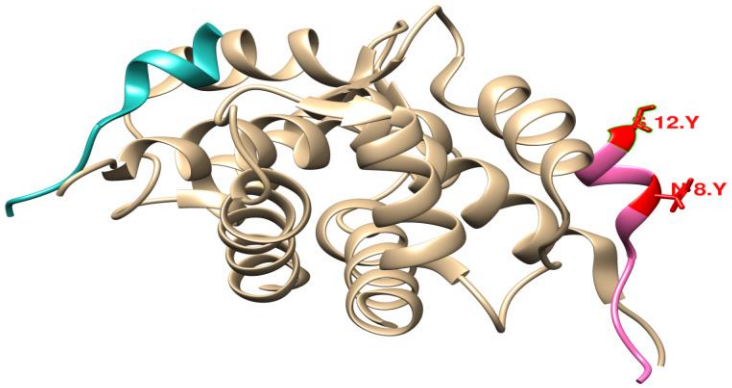


Figure 2.3. Structure of S100B-TRTK complex (1MWN). Red & cayan color are TRTK-12 peptide. The nos. are S12 &N8 that are nonessential residues.

In order to enhance the binding affinity of the peptide towards dimeric S100B. The concept of the bidentate peptide was introduced in our laboratory. The bidentate peptide is basically two helically constrained monomeric peptides that are branched through α and ε amino groups of a lysine residue. From the NMR structure of the TRTK-12 -S100B dimeric complex (pdb 1MWN), it has been observed that each S100B monomeric unit binds to a single monomeric TRTK-12 peptide. In this binding mode and the distance between two symmetrically related S12 residues

Figure 2.4: Representation of the sequence of the branched TRTK peptide with Aib substitution indicated by the letter B in the green color.

of the two TRTK-12 molecules is approximately 30Å. This distance has been covered by a branching sequence K-Ahx-K-Ahx-K where Ahx stands for 6-aminohexanoic acid. Six D-arginine residues have been added at the N-terminal end which act as cell-penetrating residues [46]. Figure 2.2 Indicates the schematic representation of the structure of the branched bidentate TRTK peptide. Figure 2.4 shows the sequence of the branched bidentate TRTK peptide with AIb substitution (indicated by the letter B in the sequence in green colour).

In figure 2.4, the residues which are highlighted in red colour are Ile 5 and Trp 7 which are the interacting residues with the protein S100B. These two residues are substituted by Ala in

the mutant branched bidentate TRTK peptide which was used as negative controls. A list of the different branched bidentate peptide with their corresponding sequences is given in table 2.1.

Specifically, at the N-terminal hexa D-Arginine tag was attached to facilitate penetration of the peptide into the cell. The substitution of the resin is first fixed while attaching the first amino acid residues during the synthesis of the peptide as the Pega resin is found in moist condition so the exact amount of the resin cannot be weighed. The weight of the resin taken should be greater than the required weight and then it is swirled in the dry DMF for at least two hours. For the first coupling amino acid taken should be equal to that of the scale of synthesis followed by the acetylation with acetic anhydride using a mixture of DIPEA and lutidine as a base in the DMF medium. Then subsequent amino acid residues were attached using a five-fold molar excess of the amino acid to be coupled with respect to the amino acid attached to the resin. When the peptide synthesis is complete, the peptide was washed with dried DMF repeatedly followed by drying with N_2 gas.

The peptide was then labelled with 5(6)- Carboxyflourescein by taking 1:20 peptide and dye molar ratio using HOBT as the coupling agent for 4 hours incubation at 25^0C. After incubation, the peptide was washed with 20% piperidine in DMF until the color of the washing becomes colorless and then dried by passing nitrogen gas. The peptide was detached using the mentioned protocol and washed repeatedly with chilled diethyl ether and finally dried. Now the peptide is ready for purification by HPLC. In HPLC a gradient is set up to 60% and the retention time of the peptide was 34 minutes. Figure 2.6 represents the HPLC chromatogram of the peptide monitored at 215nm and figure 2.7 represents the HPLC chromatogram of the peptide monitored at 490 nm

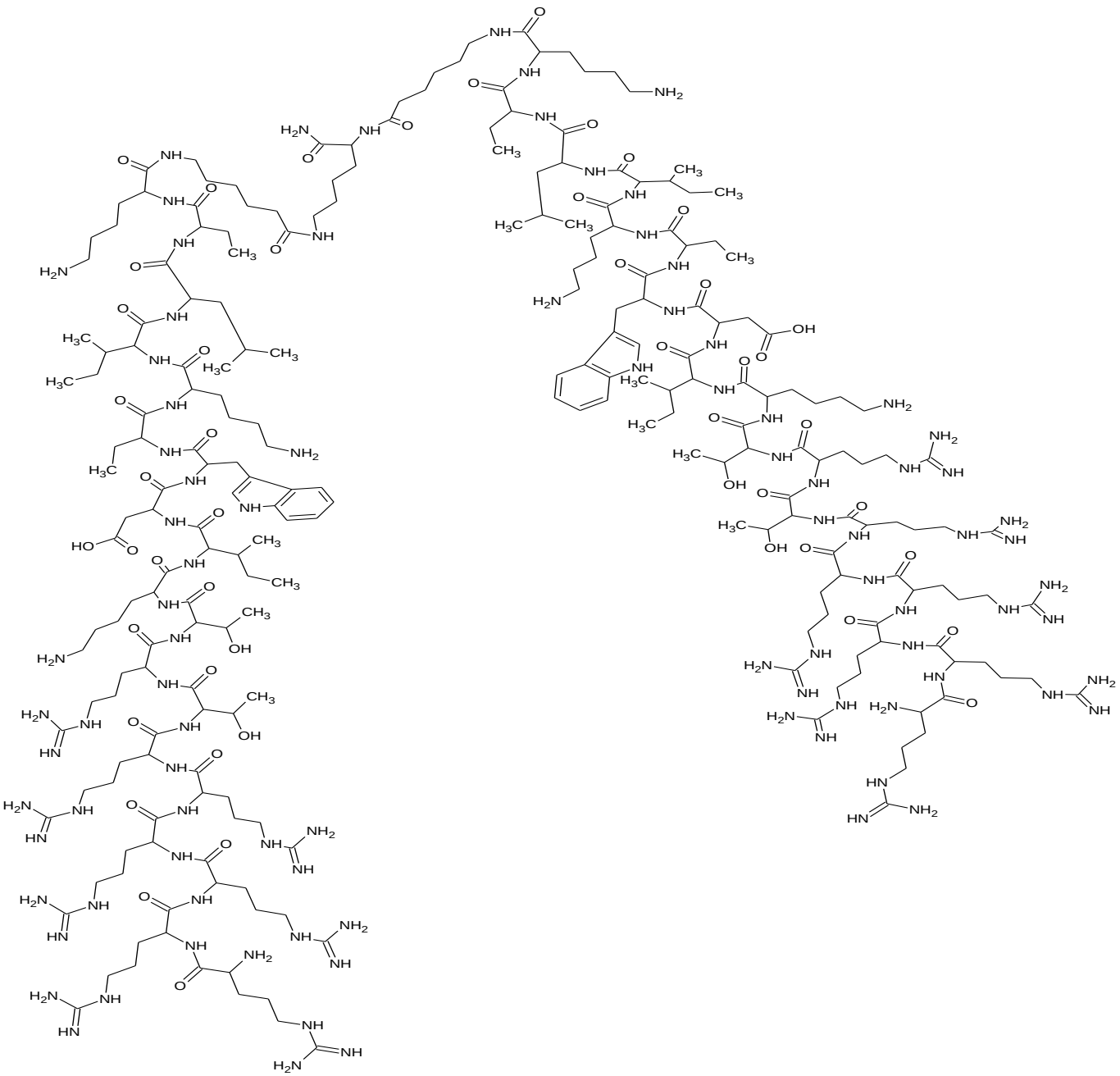
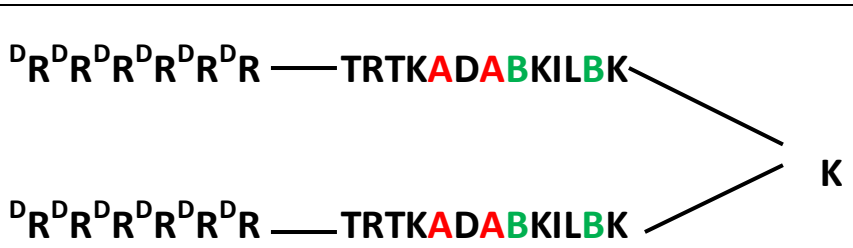


Figure 2.5: Shows the atomic structure of the branched bidentate TRTK peptide with Aib substitution.

Peptide Nomenclature	Peptide Sequence

Table 2.1: A list of different branched bidentate peptide with their corresponding sequences.

Branched dimeric wild type TRTK peptide with Aib substitution.	
Branched dimeric mutant TRTK peptide with Aib substitution.	
Branched dimeric wild type TRTK peptide with no Aib substitution.	

After the peptide was purified by HPLC, its mass was analyzed by Electro-Spray Ionization method which confirms that peptide was accurately synthesized as designed. Table 2.2 shows the mass of the different peptides. The ESI mass spectra of bidentate branched labelled TRTK peptide is shown in figure 2.8. Figure 2.9, ESI mass spectra of bidentate branched labelled TRTK peptide in m/z form. Figure 2.10 shows ESI mass spectra of

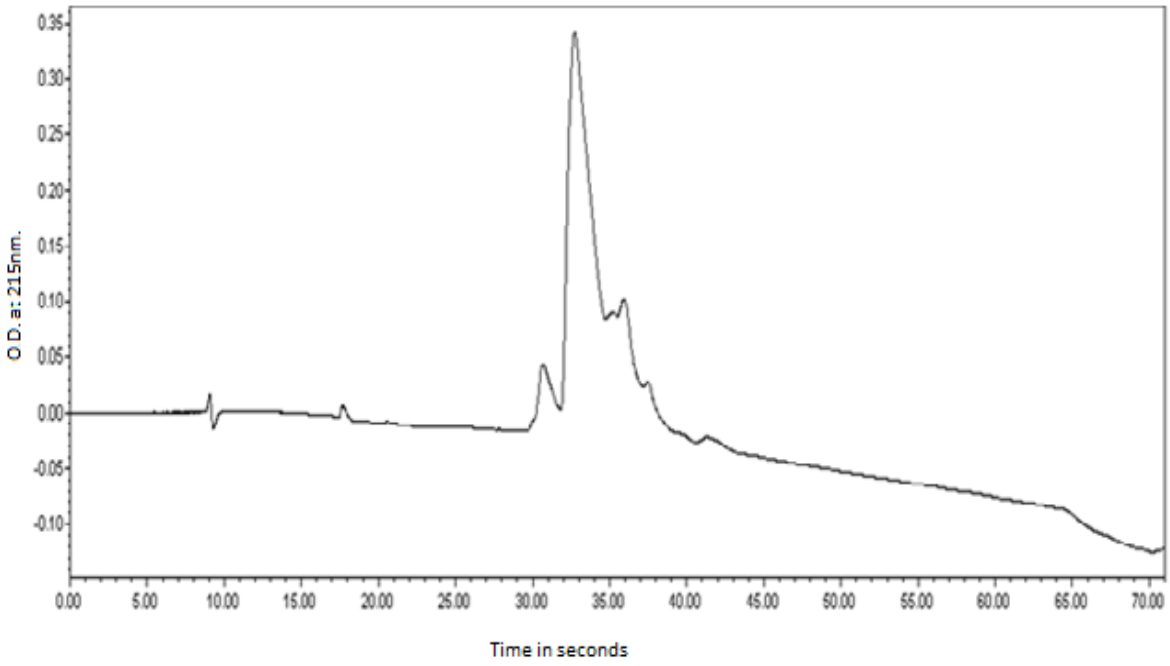


Figure 2.6: HPLC chromatogram of the labelled branched bidentate TRTK peptide at 215 nm wave length.

the mutant branched TRTK peptide unlabeled and figure 2.11 shows the ESI mass spectrum of unlabeled bidentate TRTK peptide.

In order to estimate the stability of the peptide in the biological system i.e., in the cell the peptide is digested with trypsin in the ratio 1:50 and monitored as a function of time. It was observed that the peptide with the Aib substitutions was more resistant to trypsin in comparison to that of the peptide with no Aib substitution. This is quite expected due to an increase in the helical propensity of the peptide due to Aib substitution. Up to 15 minutes, only 37% degradation of the Aib substituted Peptide occurs whereas the unsubstituted peptide undergoes 52% degradation. However, at 60 minutes, degradation becomes 68% for the Aib substituted peptide and 73% for the unsubstituted peptide (Figure 2.12).

Table 2.2: Calculated and exact mass of different peptides in Daltons.

Name of the peptides	Calculated Mass	Observed Mass
Bidentate dimeric wild type TRTK peptide with Aib substitution fluorescein labelled	6071	6072
Bidentate dimeric mutant TRTK peptide with Aib substitution.	3480	3480
Bidentate dimeric wild type TRTK peptide with Aib substitution unlabelled	5353	5353

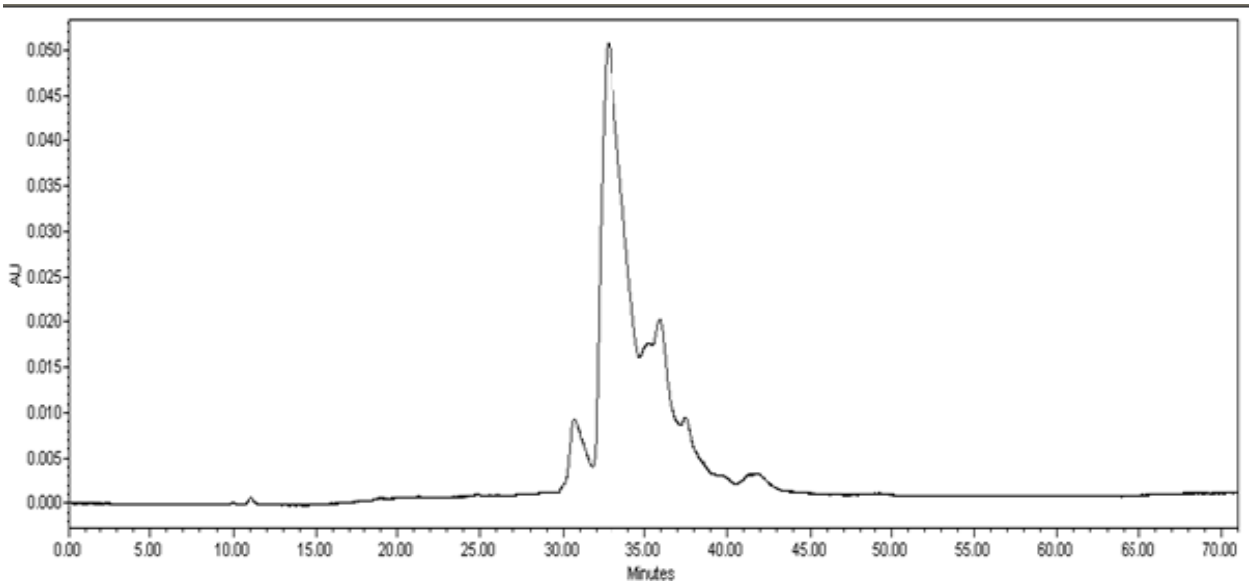


Figure 2.7: HPLC chromatogram of the labelled branched bidentate TRTK peptide at 490 nm wave length.

Thus, it can be concluded that due to Aib substitution in the peptide at proper position, the stability of the peptide increases in vitro, particularly at earlier time points. It has been reported that the stability of the peptide parallels to that of helical character of the peptide and Aib is reported to increase the helical character of peptide. The helical character of the peptide was analyzed by CD spectra of the Aib substituted and unsubstituted branched bidentate TRTK peptide; as shown in the figure 2.13, the CD spectra of branched bidentate TRTK.

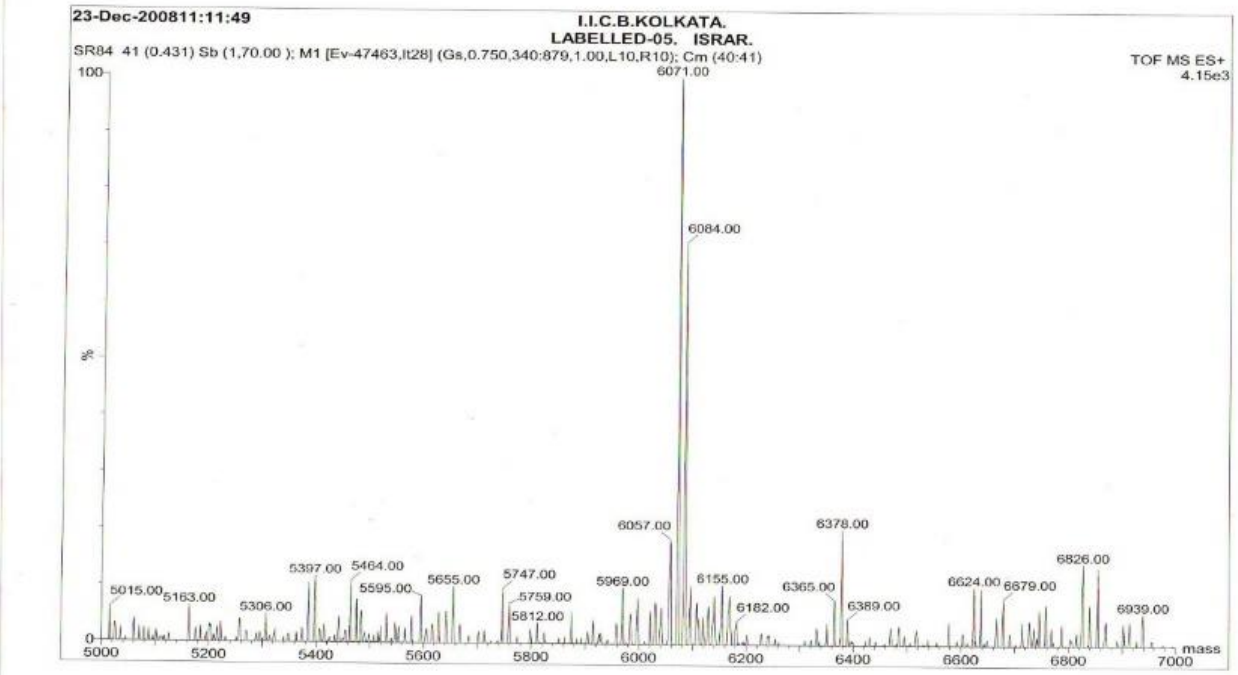
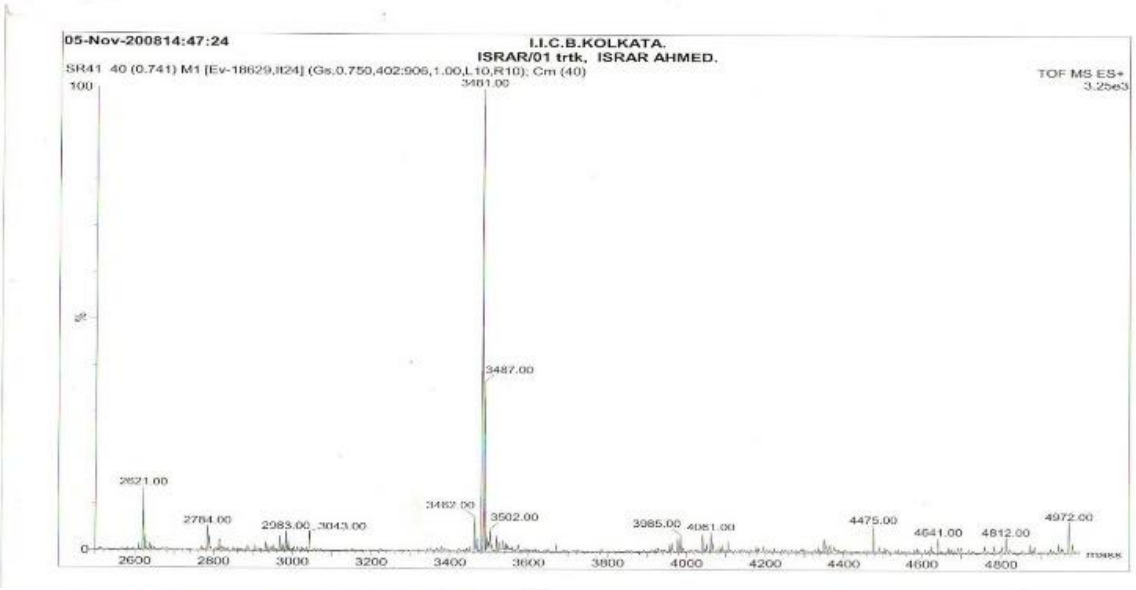


Figure 2.8: ESI mass spectra od branched bidentate TRTK peptide labeled.



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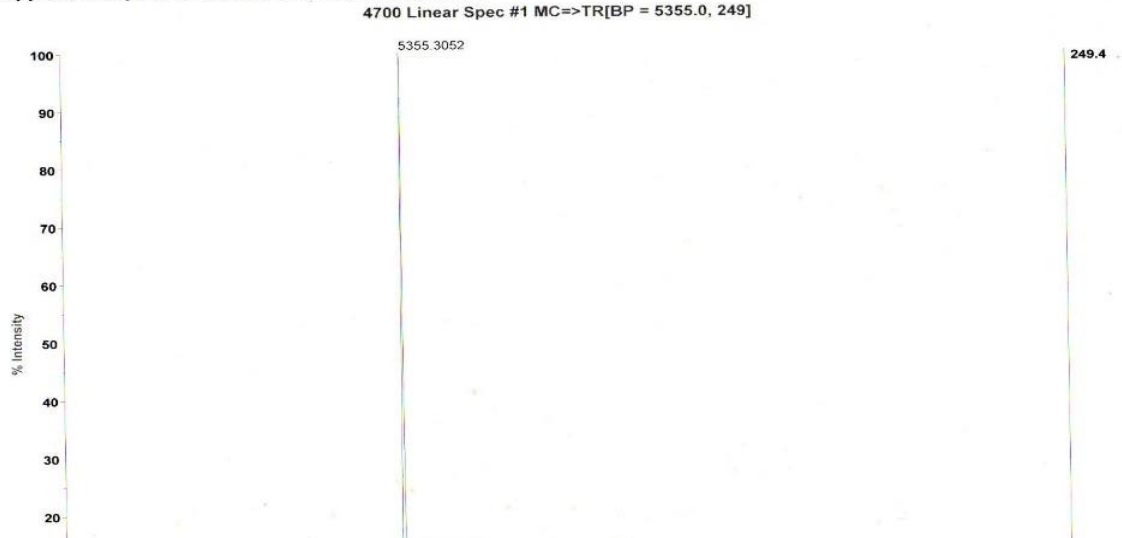

Figure 2.10: MALDI-TOF mass spectra of unlabelled bidentate TRTK peptide wild type.

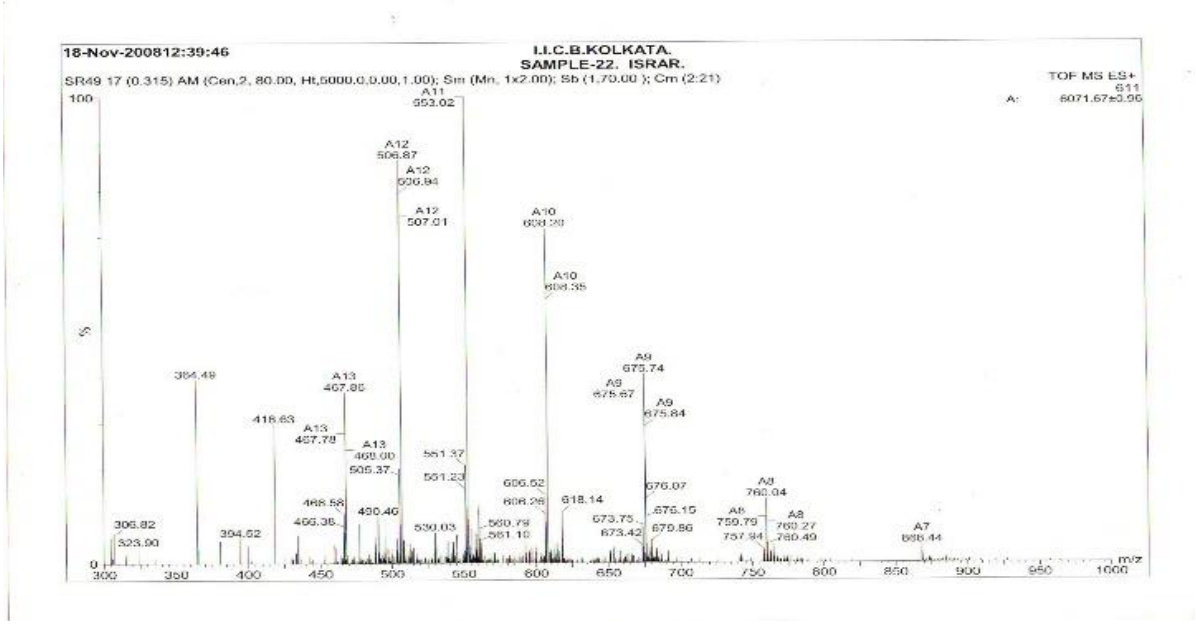

Figure 2.11: ESI m/z spectra of labeled branched bidentate TRTK wild type peptide.

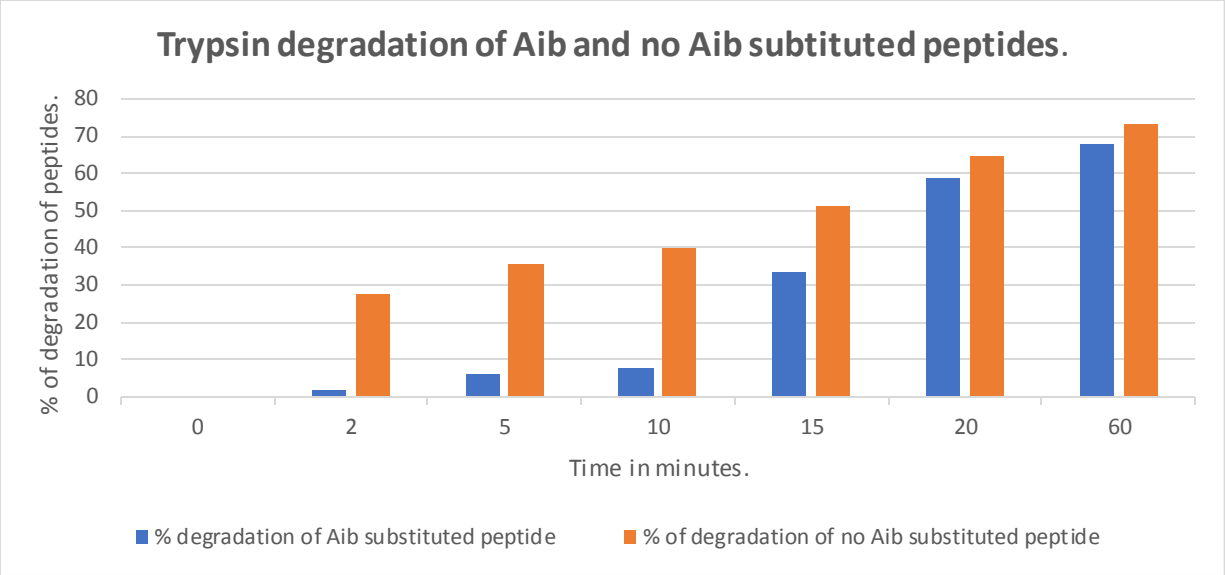

Figure 2.12: Trypsin degradation of Aib and no Aib substituted bidentate branched peptides.

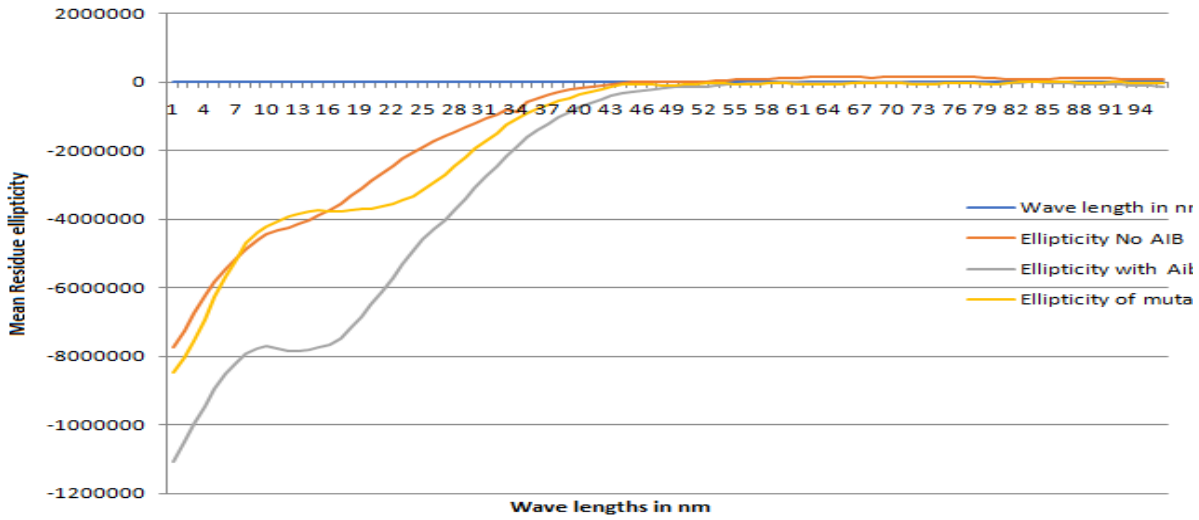

Figure 2.13: CD spectra of different peptides. (Red means bidentate branched peptide no Aib substitution, Blue means bidentate branched peptide Aib substitution and yellow means bidentate branched mutant peptide with Aib substitution)

The data that is suitably fitted into the CD spectra is given in the table 2.3.

Table 3: Raw data that is fitted into the CD Spectra

Wave length in nm	Ellipticity No AIB	Ellipticity with Aib	Ellipticity of mutant
205	-7732986	-11058622.74	-8464350
206	-7260679	-10482660.02	-8038281
207	-6743750	-9960553.64	-7535007
208	-6243576	-9448704.04	-6926723
209	-5809262	-8942230.18	-6282063
210	-5451660	-8498418.32	-5694294
211	-5146100	-8152656	-5155928
212	-4868804	-7899303.64	-4699353
213	-4628215	-7739086.8	-4406029
214	-4448870	-7695619.16	-4224606
215	-4329812	-7746606.24	-4067423
216	-4238722	-7813489.68	-3934382
217	-4144102	-7826648.7	-3839499
218	-4033058	-7777574.46	-3772549
219	-3899786	-7709305.86	-3747353
220	-3737821	-7627845.26	-3764172
221	-3547230	-7466935.84	-3767272
222	-3336916	-7185781.34	-3739009
223	-3115297	-6830850.58	-3719352
224	-2886063	-6466949.26	-3697223
225	-2655197	-6101234.04	-3634462
226	-2434099	-5706067.68	-3547955
227	-2232004	-5287056.78	-3450994
228	-2049898	-4888526.46	-3322999
229	-1883550	-4550481.46	-3153940
230	-1729405	-4268205.64	-2949586
231	-1585603	-3999715.46	-2718390
232	-1449953	-3714438.46	-2462234
233	-1320209	-3401029.52	-2200244
234	-1194282	-3063921.152	-1961245
235	-1068892	-2731037.522	-1731282
236	-941546	-2425494.312	-1487830
237	-813874	-2138017.546	-1253824
238	-879470	-1857941.492	-1061597
239	-570666	-1606033.656	-909701
240	-460434	-1387175.078	-779951
241	-364225	-1182352.788	-664751
242	-286296	-1002733.814	-562266
243	-225432	-855243.956	-466571
244	-176462	-716727.956	-378409

245	-135224	-585296.06	-303501
246	-99544.2	-477118.362	-229097
247	-66196.5	-390272.128	-145506
248	-32563	-316363.2884	-81787.4
249	-2904.21	-258951.0448	-59250.5
250	13726.28	-221263.8094	-62817.7
251	14748.19	-193042.8234	-84404.4
252	8072.086	-159404.2128	-111243
253	3594.622	-123274.9526	-113062
254	5158.237	-109882.1044	-88321.1
255	11545.8	-124967.1564	-63893.8
256	21527	-130396.9836	-53908.4
257	35311.03	-99187.35	-51531.3
258	51698.79	-56972.6202	-56281.4
259	66441.18	-34989.4714	-72826.8
260	75494.19	-25322.76956	-81157.8
261	80043.78	-9219.0643	-64063.3
262	86550.4	6364.94212	-41390.2
263	99685.2	13902.8039	-36864.1
264	116137.8	22111.47398	-49709.8
265	128592	21181.66884	-68193.1
266	135433.4	-561.148104	-80800
267	141782.3	-20504.12772	-82987.2
268	149370.7	-19924.14144	-78320.6
269	151840.2	-9624.15764	-64425.1
270	145091.6	-362.601908	-44407.9
271	135843.3	7402.45994	-36998.6
272	133549.5	15271.78476	-42159
273	139375.1	23597.94854	-44458
274	147512.3	25627.07602	-45768.3
275	151916.4	13121.9175	-51057
276	149729.9	-9946.6031	-59742
277	142781.3	-26378.98704	-65409.6
278	136574.1	-23110.66904	-59422.4
279	135548.1	-8667.01208	-50842.6
280	138902.5	-2687.945854	-46779.2
281	140573	-6991.62808	-44375.6
282	133914	-12153.49278	-51571.2
283	118456.6	-21043.7135	-65693.9
284	101411.9	-31975.19834	-64989.1
285	91144.85	-28346.27702	-43847.9
286	89902.16	-5732.28678	-17574
287	93061.32	13209.90814	-1572.06
288	94962.94	10485.06756	-1697.67
289	95349.14	-1068.265074	-8737.23
290	99254.63	-7323.209	-11781.6

291	109886.4	-16287.60174	-17651.1
292	122192.5	-36677.3878	-34049.5
293	125658.1	-53802.2528	-42978.9
294	115506.8	-50211.3904	-27719
295	98603.6	-41280.4064	-9126.49
296	84893.16	-50204.1348	-13078.8
297	76704.88	-68379.4128	-33170
298	70644.48	-80243.3082	-48322.3
299	65152.32	-95110.3624	-44812.6
300	60658.14	-123295.73	-23581.1

CHAPTER 3

BINDING STUDIES WITH S100B

S100B is a calcium-regulated protein and plays a key role in the proliferation of melanoma cells **(Dhar, Mallick et al. 2014)**. Intracellular S100B has been involved in a wide range of cellular functions as it has been found to interact with a variety of target proteins **(Donato, Sorci et al. 2009)**. It has been reported that it interacts with tumor suppressor p53. A very important negative regulator of p53-Hdm2 is also an S100B target which suggests the possibility that the two works are interconnected to regulate p53 tumor function **(Wilder, Lin et al. 2006)**. There is strong evidence that proves stimulating, proliferating and migrating actions of S100B suppressing differentiation and apoptosis **(Arcuri, Bianchi et al. 2005, Riuzzi, Sorci et al. 2006, Brozzi, Arcuri et al. 2009)**. It was also reported that the activation of p53 and induction of cell apoptosis via extrinsic pathway by reducing S100B protein level with SiRNA construct. With all this information therapeutic strategies are being thought to target Ca+2 bound S100B via small organic molecules inhibitor or via a peptidomimetic which can block the Ca+2 dependent S100B target interaction with the hopes of inhibiting the progression of cancers, specifically malignant melanoma.

Several approaches were made in order to identify the compound successfully that will identify the compound capable of binding S100B, inhibiting protein-protein complex formation. No major success has so far been reported. In this chapter, I report binding studies of a bidentate conformationally constrained peptide against the S100B protein using fluorescence

anisotropy. I show that the bidentate peptide binds it even more tightly than that of the reported TRTK-12.

3.1. Materials and methods

3.1.1. Materials

3.1.1.1. Chemicals and Reagents

All Fmoc-amino acids, Rink amide PEGA resin, HOBT, and 5(6)-carboxyfluorescein were purchased from Novabiochem (currently under Merck Biosciences, Germany). DIPEA, thioanisole, EDT, TFA, HPLC grade acetonitrile, isopropanol, and concentrated HCl were from E-Merck, Germany. Triisopropylsilane was procured from Sigma Chemicals (St. Louis, USA). DMF, Piperidine, and HPLC grade water were purchased from Spectrochem (India). Reverse-phase Hypersil Gold C18 HPLC column was from Thermo Fisher Scientific (IL, USA). Ammonium Sulphate anhydrous and PMSF were purchased by J.T. Baker (USA). Lysozyme and BSA proteins were used as reference proteins and were purchased from Invitrogen (USA) and DTT was purchased from SRL(India). Casein enzyme hydroxylates and Luria broth solid were purchased from Hi-Media. Sodium chloride, ampicillin, Calcium chloride, and agar solid were purchased from Merck (India). BL21(DE3) was stored in our laboratory at -80^0C. Acrylamide, Tris buffer pH=8, Sodium dodecyl sulphate, TEMED, glycerol, and ammonium perdisulphate was purchased from Merck (Germany now in the USA). IPTG (isopropylthiogalactosidase) was purchased from SRL(India).

3.1.2. Labelling of peptide with 5(6)-Carboxyflourescein

The peptides were labeled with 5(6)-carboxyfluorescein on the solid phase. Dry resin-bound N-terminal Fmoc deprotected peptide (3 µmol) was reacted with 10 times molar excess of 5(6)-carboxyfluorescein and HOBT in a 1:1 ratio and 20 times or even more molar excess of N-Ethyl diisopropylamine in DMF. The reaction was kept for 4 hours in darkness at 25^0C. After completion of the reaction, it was washed thoroughly with 20% piperidine in DMF until the wash solution was colorless i.e., free from the dye. The resin was then washed with DMF repeatedly.

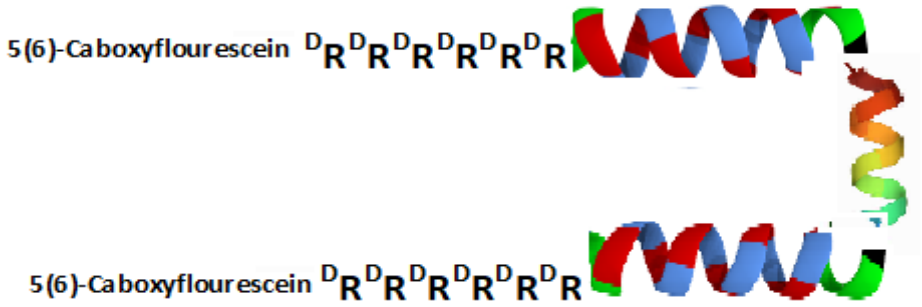

Figure 3.1: Scheme representing the labeled bidentate TRTK peptide. **R^D represents D-arginine.**

3.2. S100B purification

3.2.1. Competent Cell preparation

In a 5ml LB solution, 100 µl glycerol culture of BL21 (DE3) was added and was kept in stirring at 37^0C at 150 rpm overnight until the O.D. was raised to 0.4 which was measured at 600nm wavelength. Then it was kept in ice for 15 minutes and centrifuged at 8000 rpm for 3

minutes. The supernatant was discarded and bacterial pellets are taken and divided into four parts.

3.2.2. Plasmid preparation

In one of the competent cell preparations, 450 µl of 50 mM $CaCl_2$ was added and then re-suspended and then kept in ice for 15 minutes, and centrifuged at 8000 rpm for 3 minutes. Then again 100 µl 50 mM $CaCl_2$ was added and re-suspended and kept overnight in ice in the cold room. On the next day, 1 µl of a solution containing a plasmid that harbors the S100B gene was added and kept on ice for 20 minutes. Then a heat shock at 42^0C is given for one minute by a cold shock in ice for 5 minutes. It was then mixed with 1ml LB solution and kept on stirring for 45 minutes at 37^0C. 200 µl of this culture was spread on an ampicillin plate and kept at 37^0C for 18 hours and then finally kept at 4^0C for 24 hours.

3.2.3. Small Culture

One colony in the ampicillin plate was selected and transferred to 5 ml LB solution containing 0.1% ampicillin as an antibiotic and kept on stirring at 37^0C for 18 hours.

3.2.4. Large Culture

In 500 ml LB solution containing 0.1% ampicillin, the 5 ml culture was added and kept on stirring at 37^0C until the OD rose to 0.7; then 0.8mM IPTG was added and again kept on stirring at 37^0C for 4 hours. It was then centrifuged for 10 minutes at 4^0C at 6000 rpm; cell pellets were then collected and the supernatant liquid was discarded. One-fourth of the pellet was dissolved in 500 µl of lysis buffer [50mM Tris pH=7.5 + 50mM NaCl+ 1mM DTT] and was sonicated with three consecutive pulses of 30 seconds separated by 1-minute gaps. It was then centrifuged for half an hour at 4^0C at 13200 rpm; 400µl of the supernatant liquid was taken in an Eppendorf tube and finally kept in a beaker with a magnetic stirrer. Then ammonium sulphate was very slowly added for up to one hour with constant stirring to a final saturation of

80%. After the completion of the addition of ammonium sulphate it was kept in a cold room for 3 hours as such. Finally, it was centrifuged at 4^0C at 6000 rpm for 20 minutes and the protein then protein in the pellet or supernatant is checked by gel electrophoresis. DNA and RNA that might be present in the protein as impurities can be removed by treating with 1% streptomycin sulphate. 2.7 ml of 10% streptomycin sulphate was added in a stepwise fashion by adding 100 µl of 10% streptomycin solution with 5 minutes gap. When the addition was completed, it was kept as such in the cold room for one hour and then centrifuged at 4^0C at 12000 rpm for 20 minutes. The supernatant liquid was collected and kept on dialysis in the same lysis buffer using a 10000 MW cut-off snake skin dialysis bag purchased from Pierce (USA). For the further purification of the protein, ion exchange chromatography was performed using DEAE (DiethylAminoethyl) cellulose column and the lysis buffer as the eluting buffer solution in which the concentration of NaCl was varied from 50 mM to 400 mM whereas the concentration of the Tris buffer and DTT remained the same and the fractions containing the protein was identified by measuring their O.D. at 260 nm and as well as at 280 nm. Now all the fractions containing pure protein were mixed with each other and finally stored at -80^0C for further purposes.

3.2.5. S100B protein quantification

The protein thus prepared was pure and was quantified using the BCA kit in which the reference protein used was Lysozyme in place of BSA as per size suitability of S100B protein [10.5 KDa for monomer protein]. Figure 3.2 shows the standard curve of the protein quantification.

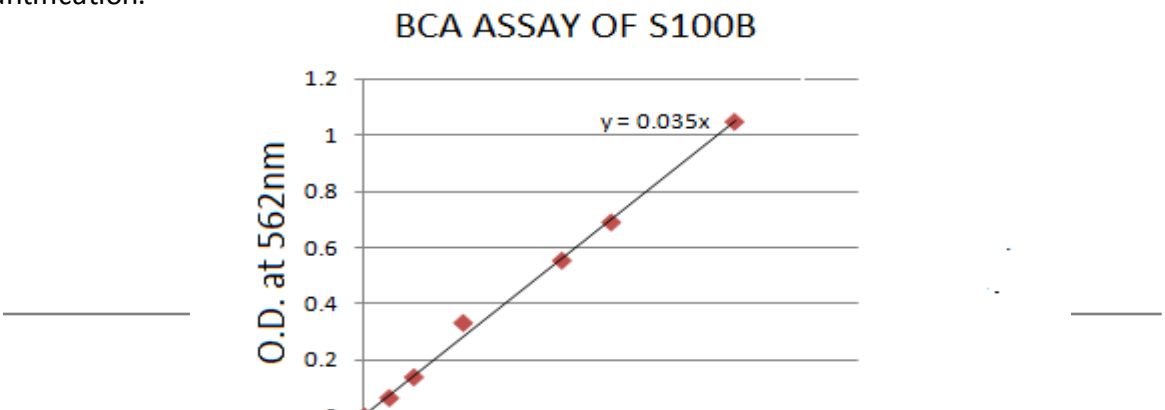

Figure 3.2: shows the standard curve of the protein quantification.

3.3. Results and Discussion

After overexpression of S100B protein from the plasmid, it was purified as a first step by Ammonium Sulphate precipitation. Figure 3.3 shows SDS gel electrophoresis of the S100B protein after the Ammonium Sulphate precipitations. The protein is already substantially pure at this stage but requires further processing to obtain a pure preparation.

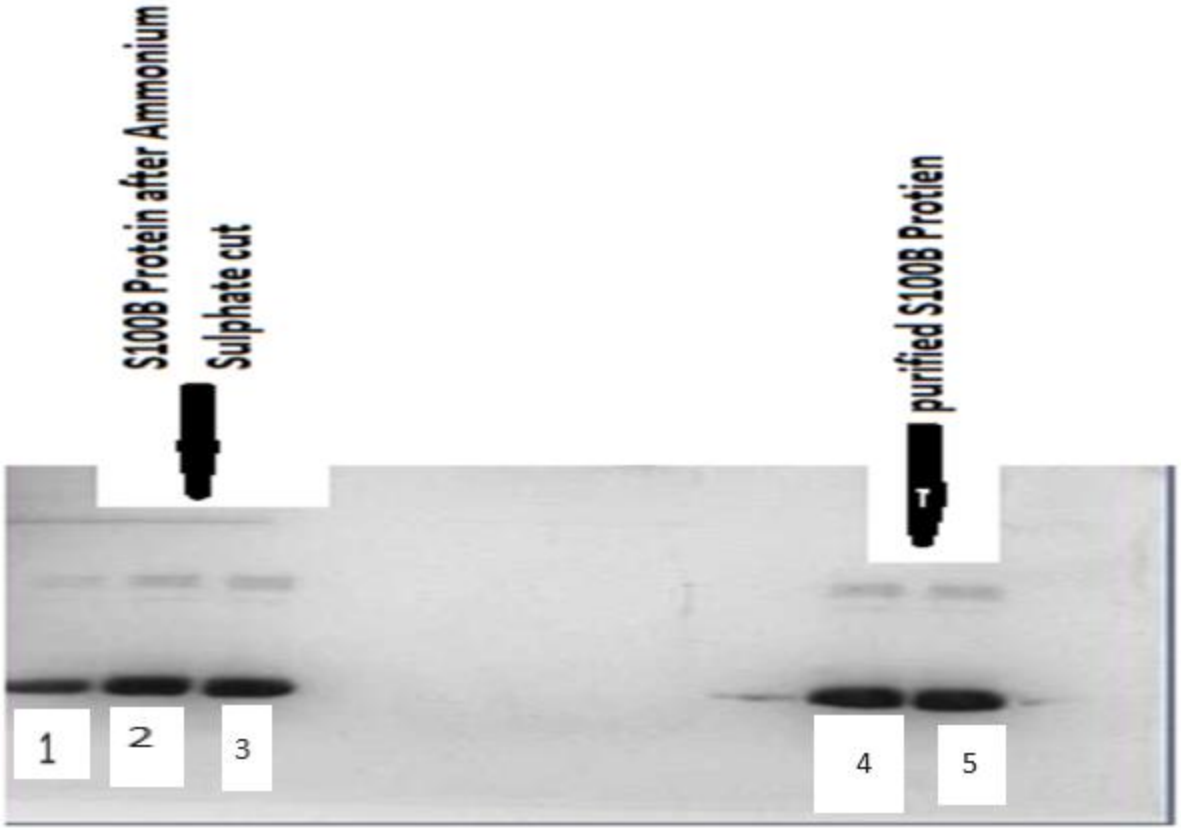

Figure 3.3: This is the profile of 15% SDS page gel electrophoresis .In this profile, lanes 1,2 &3 is the gel electrophoresis of S100B protein obtained after Ammonium sulphate whereas the lanes 4 & 5 are the purified S100B protein used as marker.

The protein-bound nucleic acids were removed by streptomycin sulphate precipitation. Figure 3.4 shows the purity of the preparation after the precipitation by streptomycin sulphate.

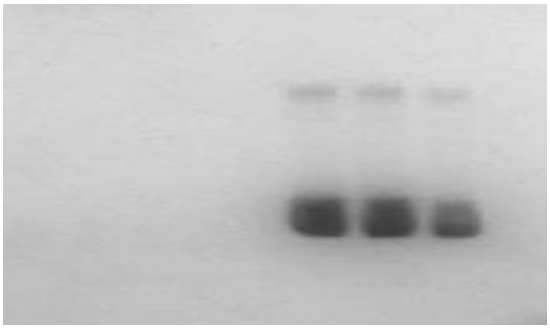

Figure 3.4: in this 15% SDS page gel profile the first two lanes from right in the picture of gel electrophoresis of S100B after streptomycin sulphate precipitation, whereas the last one is the purified S100B protein used as marker.

The protein was finally purified by Ion exchange chromatography and the purity of different fractions is shown in figure 3.5.

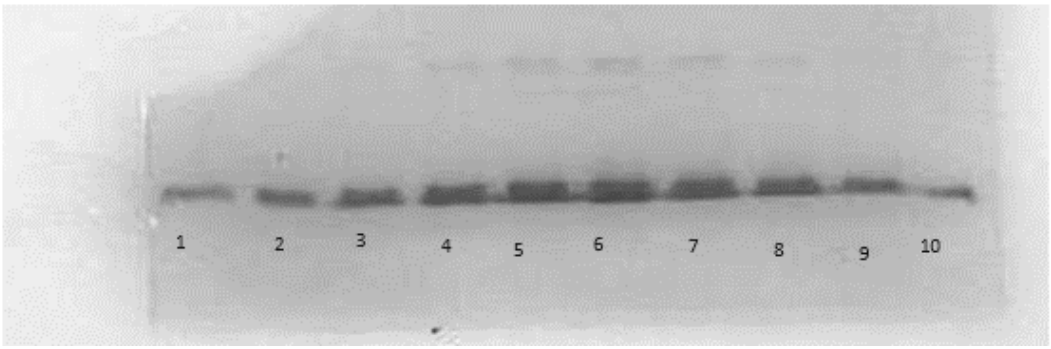

Figure 3.5: The profile of gel electrophoresis of 15% SDS page lane 1 to 9 are the fraction S100B protein after ion exchange chromatography including the last one i.e. 10 is purified S100B protein using as marker in it all the fractions shows pure protein.

The purest protein fractions were pooled together and used for further studies.

A conformationally constrained helix monomer TRTK was previously designed in our laboratory which was being used as a peptide-based inhibitor of S100B-p53 interactions. It was derived from the Actin capping CapZ (265-276) protein. The idea was that the TRTK peptide interacts with the S100B protein more tightly than that of p53. Thereby p53 will be released from the

p53- S100B complex. In the structure of the S100B-p53 complex, it was observed that N8 and S12 are the non-interacting residues and these residues are pointing away from the protein. So, these two residues were substituted by Aib to improve the stability and efficacy of the peptide in the cell as the helicity of the peptide and its resistance towards the trypsin also Improved. Since S100B

Figure 3.6: Schematic diagram of branched bidented TRTK peptide containing hexa D-arginine

is a dimeric protein of 21 KDa. a bidentate peptide was designed to further enhance the binding affinity of the peptide towards the dimeric S100B.

A covalently linked dimer was designed using the concept of double Fmoc-protected lysine as a branching point. In this bidentate helically constrained peptide, the branching occurs at the α and ε amino groups of lysine. The distance between the two symmetrically related S12 residues of two TRTK-12 molecules was calculated and found to be 30Å approximately. This distance is approximately covered by using an additional sequence K-Ahx-K-Ahx-K, this sequence was being used to connect the two terminal Aib residues where Ahx is 6-aminohexanoic acid. The interaction of bidentate branched helical TRTK peptide with S100B protein was measured by fluorescence anisotropy and it was found that it binds to the protein very tightly with a dissociation constant (KD) of 11.2±1.9 nanomolar which is tighter in comparison to that of TRTK-12 monomer [KD of TRTK-12 with S100B protein is 2.9 µM **(Garbuglia, Verzini et al. 1999)**]. Figure 3.7 shows the binding isotherm of bidentate branched TRTK peptide with S100B protein as determined by fluorescence anisotropy. From this study, it was evident that bidentate branched peptide binds S100B protein much more tightly. In order

to determine target specificity, we compared the binding constant of the interaction of bidentate branched TRTK peptide with S100B with that of the binding constant with another member of the S100 protein, in this case, S100P. However, it was observed that it binds S100P protein significantly weakly in comparison to S100B; its dissociation constant of S100P-peptide protein was found to be 62.8±4.5 nM.

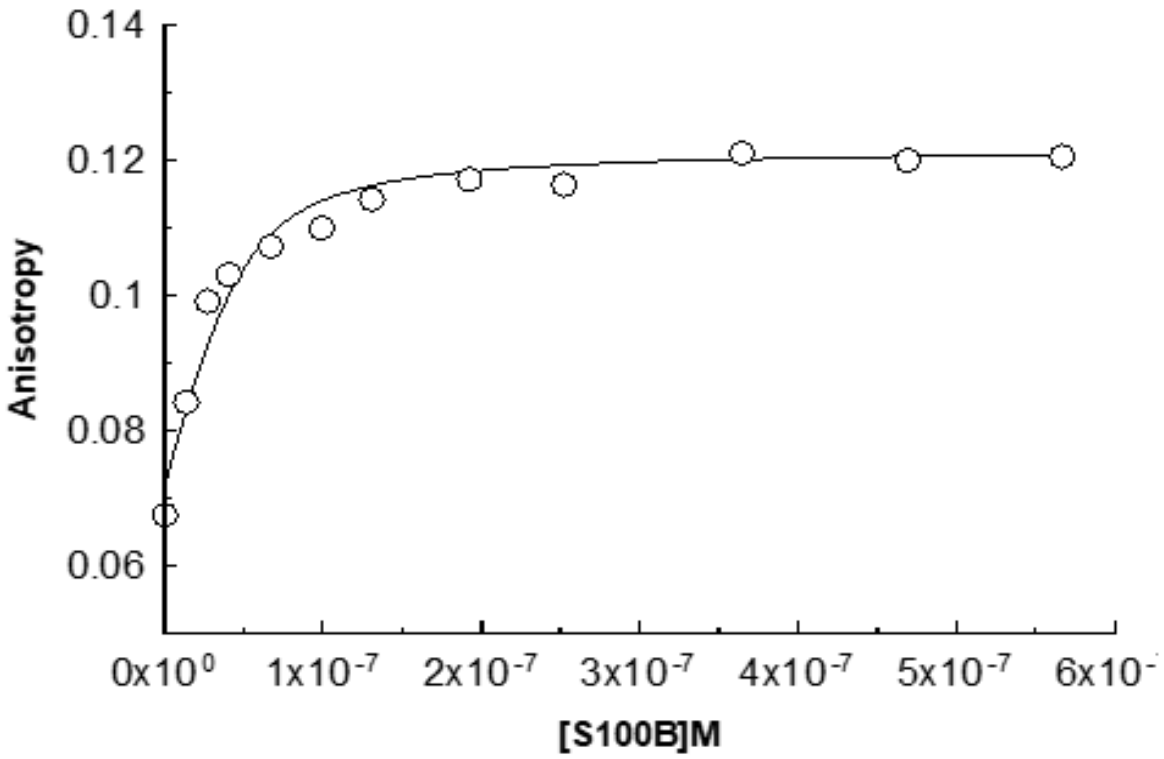

Figure 3.7: Binding isotherm of branched bidentate TRTK peptide with S100B. 50nM Peptide labelled with 5-(6)-carboxy fluorescein was taken and titrated with increasing concentration of S100B. At each point fluorescence anisotropy was measured which is an average of three independent experimentation using 50mM Tris, 50mM NaCl and 1mM DTT at pH=7.5

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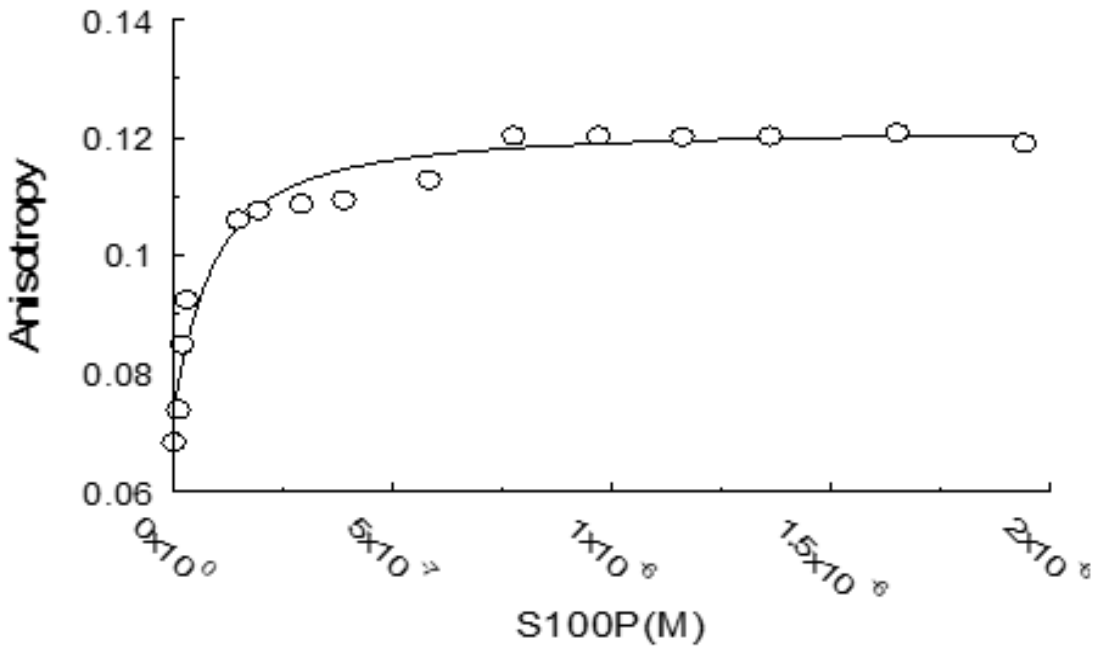


Figure 3.8: Binding isotherm of branched bidentate TRTK peptide with S100B. 50nM Peptide labelled with 5-(6)-carboxy fluorescein was taken and titrated with increasing concentration of S100B. At each point fluorescence anisotropy was measured which is an average of three independent experimentation using 50mM Tris, 50mM NaCl and 1mM DTT at pH=7.5

To determine the stoichiometry of the interaction of the peptide with the protein, another titration was designed and performed in which a high peptide concentration was used I.e.,340 nM, and the protein concentration is successively increased and reached up to 1.6 µM. Figure 3.9 shows the stoichiometric titration of bidentate branched TRTK peptide with S100B protein.

From this data it can be concluded that the stoichiometric ratio through which the peptide interacts with the protein is 1:1 i.e., one molecule of the peptide interacts with one molecule of the protein though both are dimeric in nature.

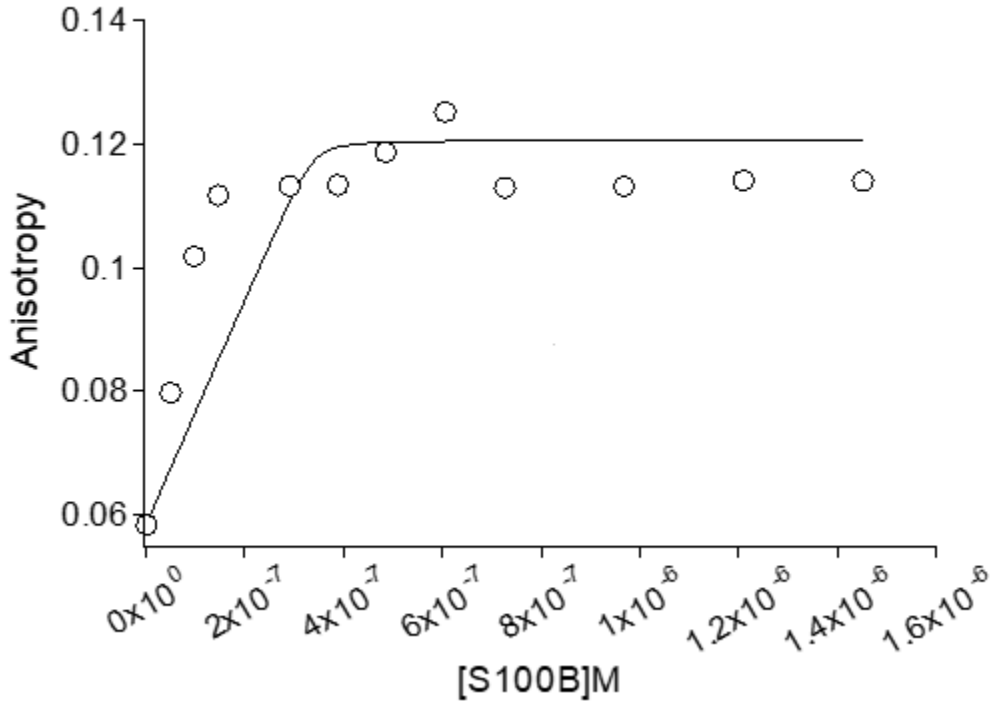


Figure 3.10: : Binding isotherm of branched bidentate TRTK peptide with S100B. 340nM Peptide labelled with 5-(6)-carboxy fluorescein was taken and titrated with increasing concentration of S100B. At each point fluorescence anisotropy was measured which is an average of three independent experimentation using 50mM Tris, 50mM NaCl and 1mM DTT at pH=7.5

From the NMR structure of the S100B-TRTK-12 complex, it is evident that Ile5 and Trp7 are the two residues that directly interact with the S100B protein when these two residues were substituted by Ala the binding constant of the substituted bidentate branched TRTK peptide with the S100B protein decreases; the dissociation constant was found to be 132±9.8

nanomolar indicating that the wild type bidentate branched peptide binds S100B protein approximately 10 times stronger than that of the mutant bidentate branched TRTK peptide. Figure 3.10 shows the binding isotherm of the mutant bidentate branched TRTK peptide with the protein S100B.

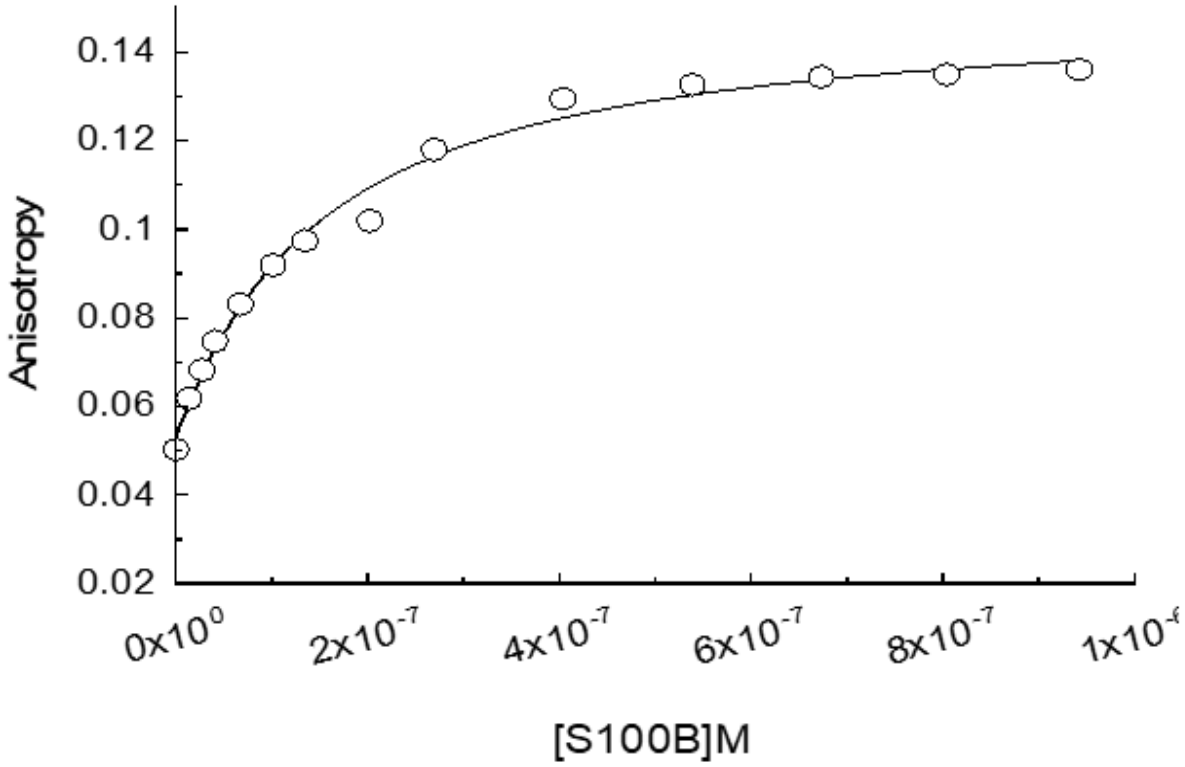


Figure 3.10: Binding isotherm of branched bidentate TRTK peptide with S100B. 50nM Peptide labelled with 5-(6)-carboxy fluorescein was taken and titrated with increasing concentration of S100B. At each point fluorescence anisotropy was measured which is an average of three independent experimentation using 50mM Tris, 50mM NaCl and 1mM DTT at pH=7.5

3.4. Conclusion : -

TRTK -12 derived from Cap Z protein (265-276) binds to S100B protein in the same region where p53 binds even with even tighter affinity than p53**(Fernandez-Fernandez, Rutherford,**

TJ, &Fresht AR.). A branched bivalent helical TRTK peptidomimetics was designed and synthesized which shows greater binding affinity and stability than that of unmodified normal TRTK. We have chosen substitution of alpha aminoisobutyric acid(Aib) substitution as an alternative due to its simple synthesis methodology and possibly better scaling up potential for branched TRTK molecule. Using the crystal structure as a guide we replaced N-8 and S-12 residues of the target peptide that are not interacting with S100B. This shows much more tighter binding with S100B protein. The specificity of the peptidomimetic was ascertained by substituting the residues I-5 and W-7 on the peptidomimetic. Reduced binding of the mutant peptide indicate specificity of peptidomimetic -S100B interaction. Impaired binding with S100P, a closed paralog of S100B supported specific binding to the target. In summary a peptidomimetic targeting S100B was synthesized which showed tight affinity and high specificity for the target.

CHAPTER 4

PHARMACOKINETICS & TISSUE DISTRIBUTION STUDIES OF THE BIDENTATE TRTK PEPTIDE

The development of therapeutic molecules is a long process starting with the discovery of new molecules that can target specific proteins. In the initial phases, finding a good ligand for the targeted protein is a major task; this is usually followed by uncovering the specificity of that particular molecule toward the target. Once these are established, it is important to find out whether the particular molecule in the targeted tissue is in sufficient quantities and does not cause any side effects elsewhere. Without adequate concentrations in the targeted tissue, it is difficult to have an appropriate effect of the molecule on the tissue. In the case of antitumor compounds, it is important that there is a sufficient accumulation of the drug in the tumor. For therapeutic peptides, delivery to the targeted tissue assumes even more importance as the peptides generally tend to have a shorter half-life in the plasma. Thus, in this small chapter, we have attempted to determine the distribution of the bidentate peptide in different tissues in wild-type mice as well as in tumor-bearing mice.

Technetium-99m (^{99m}Tc) based radiopharmaceuticals are used routinely in nuclear medicine for the diagnosis of diseases such as cancer, inflammation, myocardial infractions and others (Charron, Orenstein et al. 1994, Hung, Huang et al. 2008, Akutsu, Kaneko et al. 2009). Clinical imaging using ^{99m}Tc- radiopharmaceuticals targeted to specific receptors produced successful results in many cases thus demonstrating the utility of such approaches for developing site-specific radiopharmaceuticals (Nock, Nikolopoulou et al. 2003, Hung, Huang et al. 2008). Various biomolecules attached to small receptor binding peptides are currently the agents of choice for receptor imaging and tumor targeting. Attachment of suitable and biocompatible ligating groups to a bio reductive pharmacophore is required for technetium radiolabeling. 2-Nitro, 4-nitro, and 5-nitro imidazoles coupled with different technetium binding

chelators have been extensively studied for in vivo assessment of tumor hypoxia (Chu, Hu et al. 2004, Chu, Li et al. 2004, Mallia, Subramanian et al. 2008). However, slow blood clearance and significantly high hepatobiliary uptake are the limitations, and ideal potentially active compounds remain to be developed.

As a part of my ongoing research program, I radiolabeled peptides with fac-$[^{99m}Tc(CO)_3(H2O)_3]^+$ synthon core. The fac-$[^{99m}Tc(CO)_3(H2O)_3]^+$ precursor **(Halder, Nayak et al. 2011)**was attached to the peptides via histidine (His)) residue. In this study, 5-nitroimidazole and 2-nitroimidazole were labeled with either fac-$[^{99m}Tc(CO)_3(H2O)_3]^+$ core or $^{99m}TcO_4^-$ under mild conditions. ^{99m}Tc-radiolabeling was mediated through the amino acid histidine coupled to the pharmacophore. I therefore took up the synthesis of 2-nitroimidazole conjugated with peptide via the β-COOH function of the aspartic acid. The terminal His residue of the peptide acts as chelator for the fac-$[^{99m}Tc(CO)_3(H2O)_3]^+$ core.

4.1. Materials and Methods

4.1.1. Chemicals

All chemicals and solvents were either of analytical or HPLC grade and used as supplied without further purification. Rink Amide MBHA resin (100–200 mesh) and all side chain protected amino acids, HOBT (N-hydroxybenzotriazole) and TBTU [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate], were purchased from Novabiochem (Darmstadt, Germany). 2-Methyl-5-nitroimidazole, 2-nitroimidazole, N, N0 -dicyclohexylcarbodiimide (DCC), sodium methoxide, N,N-diisopropylethylamine (DIPEA), picryl sulfonic acid, thioanisole, and TFA (trifluoroacetic acid) were purchased from Sigma (St. Louis,

MD, USA). $^{99m}MoO_4^-$ was purchased from Bhabha Atomic Research Centre, Mumbai, India and 99mTcO4 was obtained by 2-butanone extraction from a 5 N NaOH solution of $^{99m}MoO_4^-$. Mass spectrometry (MS) was performed on a Waters Q-Tof micro mass instrument and MALDI-Tof. HPLC analyses of the ligands and the radiopharmaceuticals were performed on a Waters HPLC system using XTerra Prep C-18 column (7.8 9 300 mm, 10 lm particle size). The column was eluted at a flow rate of 2 mL/min using a linear gradient of 0.1% TFA in H_2O (solvent A) and CH_3CN (solvent B) changing from 100% A to 85% A in 25 min. Radioactivity in the eluate was monitored using a Berthold LB500 HERM radio HPLC monitor procured from Berthold Technologies GmbH (Bad Wildbad, Germany). Various countings were performed in a well-type gamma counter from Electronic Corporation of India Limited (Model LV4755 Hyderabad, India).

4.1.2. ^{99m}Tc radio labeling

Preparation of ^{99m}Tc(CO)3-complexes of ligands [$^{99m}Tc(CO)_3(H2O)_3$] + precursor. The synthon was prepared using the modified procedure reported as mentioned in **(Schutte, Kemp et al. 2011)**. An aqueous solution of $NaBH_4$ (5.5 mg), Na_2CO_3 (4 mg), and Na/K tartrate (15 mg) in 0.5 mL double-distilled water was taken in a sealed glass vessel was purged with carbon monoxide for 6–8 min. Freshly extracted $^{99m}TcO_4^-$ (185–370 MBq, in 0.5 mL distilled water) was added and the mixture and heated at 80 °C for 20 min, cooled to room temperature, and the pH was adjusted between 6.5 and 7.0 with 0.5 M phosphate buffer (pH 7.5): 1 M HCl (1:3 v/v). The synthon was characterized by HPLC.

4.1.3. Labeling of peptide with $[^{99m}Tc\ (CO)_3(H2O)_3]^+$

Under optimized conditions, 0.3 mL of freshly prepared [$^{99m}Tc\ (CO)_3(H2O)_3]^+$ precursor was added to the ligand solution (4–5 mg dissolved in 0.2 mL of N_2 purged water) taken in a

separate vial, and the final pH of the mixture was kept between 6 and 6.5. The mixture was heated at 45 °C for 10–15 min. Thus the full sequence of the peptide is **[$(H_20)_3(CO)_3Tc^{99m}$]NH2-HTRTKIDWAibKILAibK-K-KAibLIKAibWDIKTRTH-NH2-[^{99m}Tc $(CO)_3(H2O)_3$].** Figure 1 shows the HPLC chromatogram of the Tc labeled branched bidentate TRTK peptide. Methanol-water was used as HPLC solvent system and an isocratic 80% methanol -water was run.

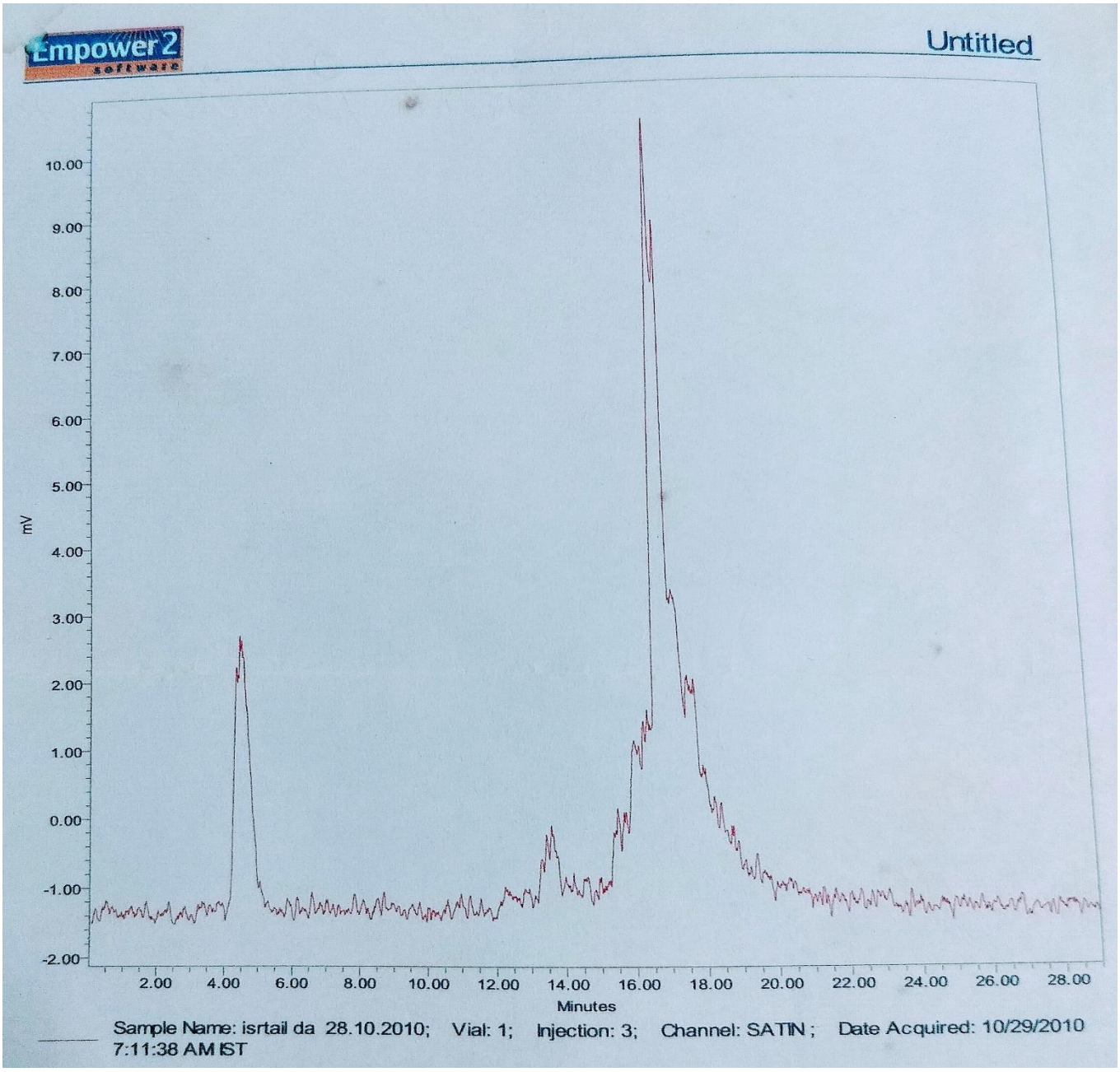


Figure 4.1: shows the HPLC chromatogram of the ^{99}Tc labeled branched bidentate TRTK peptide. Time is plotted at X-axis while milli-absorbance is plotted at Y-axis.HPLC performed using an isocratic gradient of 30% methanol in water.

4.1.4. Preparation of ^{99m}Tc complex by direct reduction using $SnCl_2.2H_2O$

To an aqueous solution of the peptide (2 mg dissolved in 0.5 mL H_2O), adjusted to pH 4.0 with 0.1 N $NaHCO_3$, was added aqueous $^{99m}TcO_4^-$ (74–148 MBq, 0.1 mL) followed by freshly prepared $SnCl_2.2H_2O$ solution (16 mg, 20 mLi.e.,0.8mg/ml) under nitrogen atmosphere. The mixture was thoroughly purged with dry nitrogen gas and allowed to stand for 15 min at room temperature. The radiochemical yields of the complexes were evaluated by thin-layer chromatography (TLC). Precoated silica gel plates were used as stationary phase and were developed either in acetone or in methanol/TFA 9:1 to determine the presence of potential radiochemical impurities, namely pertechnetate and reduced hydrolyzed technetium in the complexes. HPLC analysis (methods described under synthesis) of the radiolabeling mixture revealed the formation of mainly one complex with high radiochemical purities (>90%).

4.1.5. Biodistribution studies in mice

All animal experiments were approved by the Social Justice and Empowerment Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, New Delhi. Biodistribution studies were performed in EAT-bearing Swiss albino mice (body weight 25–30 g). For the induction of the tumor, freshly collected EAC cells (approximately 2 X 10^7 cells) suspended in saline were injected intramuscularly in the thigh of the right hind leg of each mouse. After 12–14 days, when tumors had reached an approximate

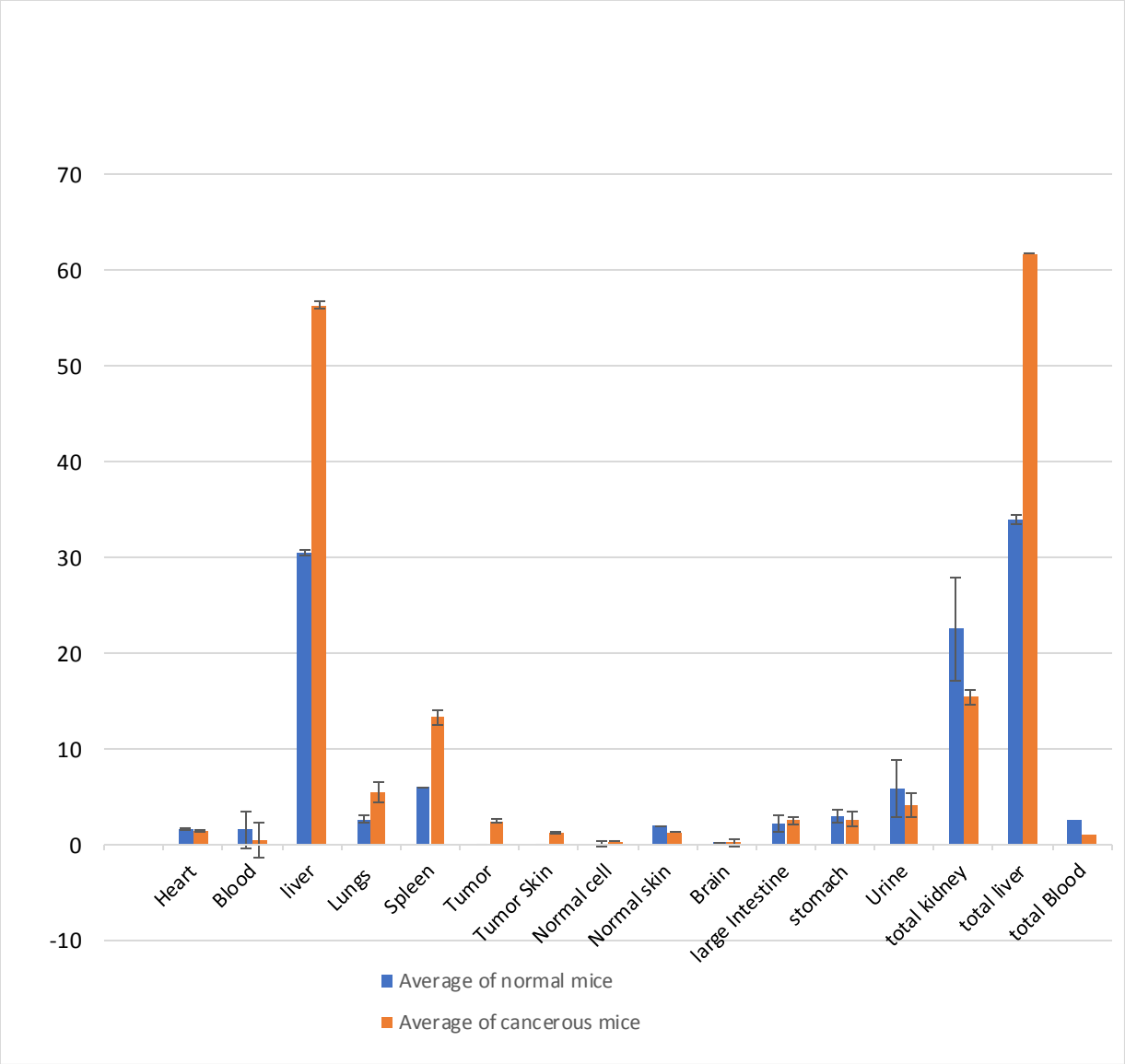


Figure 4.2: shows the diagrammatic representation of the biodistribution of the ^{99}Tc labeled bidentate branched TRTK peptide in normal and as well as cancer bearing mice. In the vertical axis Y-counts are plotted. This is the average data of 4 mice in both normal and cancerous mice.

volume range of 1 0.1 cm^3, the mice were used for biodistribution studies. All animals were well hydrated by intraperitoneal administration of saline (0.9%, 2 mL) for 1 h. After another hour, 99mTc-labeled complexes (0.03 mL, 8–12 MBq/kg) were injected via the tail vein. After different time intervals (2, 4, and 6 h) the animals were sacrificed, the organs or tissues of

interest were excised, washed with normal saline to free from adhering tissues, and transferred into counting vials. The urinary bladder was clamped, dissected, and carefully transferred into counting vials. Blood samples were obtained by puncture of the heart. The samples were counted against suitably diluted aliquots of the injected solution as a standard and the results were expressed either as % ID/g of tissue or % ID/organ.

4.2. Results and Discussion

To assess the potentiality of our approach for the design of potential radiopharmaceuticals for tumor targeting, evaluation in mice bearing Ehrlich Ascites tumor was performed. Tissue distribution data are summarized in Table 4.1. Figure 4.2 shows the diagrammatic representation of the biodistribution of the Tc labeled bidentate branched TRTK peptide. 4 hours study of the tumor uptake was done and this uptake was compared with the normal mice tissues and observed that the uptake was not significant in the heart, stomach, brain, and large intestine. The data were found to be almost similar in tumor-bearing mice tissues with that of normal mice tissues. The most significant uptake was observed in the liver and kidney. However, some localization in the tumor was also observed. Since no significant change in the brain uptake is observed when compared with the normal cell indicating that the peptide when chelated with the ^{99}Tc cannot cross the blood-brain barrier. A significant decrease in the accumulation of the peptide in the kidney was observed i.e., quite expected due to an increase in the size or molecular weight of the peptide when labeled with Tc complex that might also be the reason for low renal clearance. From the above data it is evident that the peptide mostly accumulates in the liver and it is very rarely or almost no amount was found in

the brain indicating that the peptide has no capability of crossing the blood brain barrier. Renal secretion of the peptide was also poor.

Table 4.1: Tissue distribution of the Tc-labeled bidentate branched peptide in percentages of total.

organ	Normal Tissue	Tumor Tissue
Heart	1.545±0.103	1.320±0.15
Blood	1.47±0.051	0.455±0.094
Liver	30.435±1.88	56.310±1.851
Lungs	2.565±0.231	5.45±0.382
Spleen	5.96±0.381	13.235±1.008
Tumor	NA	2.36±0.803
Tumor Skin	NA	1.20±0.214
Normal Cell	0.595±0.017	0.305±0.096
Normal skin	1.885±0.242	1.270±0.043
Brain	0.0674±0,012	0.0690±0.005
Large Intestine	2.0865±0.121	2.465±0.428
Stomach	2.96±0.872	2.60±0.336
Urine	5.715±0.68	4.08±0.793
Total Kidney	22.47±2.30	15.37±1.254

Total Liver	33.875±5.427	61.74±0.736
Total Blood	2.44±0.475	0.965±0.079

In chapters 2, 3, and 4, we have shown the design and development of a bidentate peptide that can bind with high affinity to an Important drug target, S100B. we have also shown that a radio-labelled version of the peptide accumulates mostly in the liver but also significantly in an implanted tumor in a mouse model. In a parallel experiment in our laboratory, we have been able to show that the peptide is capable of inhibiting tumor growth in another mouse model **(Dhar, Mallick et al. 2014)**. Taken together these experiments bode well for further developing the peptide as a therapeutic molecule.

CHAPTER 5

DESIGN AND DEVELOPMENT OF KLF1 MIMICKING PEPTIDE

In chapters 5, 6, and 7, we have reported studies that modify different peptides to achieve important goals in different therapeutic areas. in chapter 5 we have designed and developed a modified peptide that can bind tightly to a specific DNA sequence that is the target of a zinc finger transcription factor.

Among the Indian population, monogenic disorders such as β-hemoglobinopathies, including β-thalassemia and sickle cell diseases, occur at above average frequency. These diseases often show poor outcomes due to largely ineffective and unaffordable nature of the available therapies. The underlying cause of these diseases is due to a defective β-globin gene expression which is one of the subunits of the most abundant adult hemoglobin [a tetramer containing two α-globin and two β-globin subunits]. However, during early development, HbF, or the fetal hemoglobin [a tetramer containing two α-globin and two γ-globin subunits], is normally expressed during fetal development and in the first month of life; later, it is replaced by HbA (adult hemoglobin, a tetramer of two α-globin and two β-globin chains). One of the possible therapeutic strategies against β-hemoglobinopathies is to upregulate the fetal hemoglobin, which is functionally close to HbA. Hydroxyurea, a drug of choice in many β-hemoglobinopathies, is a single organic molecule that acts by the upregulation of HbF. The most viable option in this regard is the allogeneic stem cell transplantation though it is not free from post-treatment complications. These diseases are in dire need for effective and affordable therapies.

Targeted interference with the gene regulatory network of the ϒ-globin gene, with the end goal to upregulate their expression could be a new approach. Small molecules can be a good choice, but may require a lot of time for design/screening to arrive at a lead molecule. Developing peptide-based transcription factor mimics against critical transcription factors in the γ-globin gene regulatory network could be an alternative approach. Four main regulators of ϒ-globin gene expression that comprise the main nodes of the regulatory network, including Klf1, LRF/ZBTB7A, BCL11A, and BP1/DLX-4. ZBTB7A and BCl11A repress ϒ-globin via a number of pathways. Notably, a naturally occurring genetic mutation in the BCL11A binding domain in ϒ-globin promoter creates a phenotype with high constitutive HbF expression that is resistant to β-hemoglobinopathies. BP1 is also hypothesized to be repressed ϒ-globin, in addition to its well-known role in β-globin repression. Thus, interference with the DNA-binding activity of any of these four factors, combined with active downregulation of these factors' expression, has the potential to reactivate ϒ-globin expression in adult erythrocytes.

The ϒ-globin gene regulatory network that controls ϒ-globin gene expression as shown in figure 5.1. In gene regulatory networks, conformationally-constrained protein-protein interaction inhibitors have been used to alter gene expression through disruption of protein-protein interaction **(McGrath, Tortorici et al. 2017, Wachter, Morgan et al. 2017)**. There are a number of approaches through which enhanced ϒ-globin expression can be achieved via disruption of repressive multi-protein regulatory complexes of the ϒ-globin gene. One of the key repressors of the ϒ-globin gene is BCL11A. There are two different modes through which BCL11A represses the ϒ-globin gene in adulthood. In one mode, BCL11A binds one or more TGACCA motifs through its C-terminal Zn finger motif near the ϒ-globin gene causing the

switching of a DNA loop between the LCR region from near the ϒ-globin gene to that of the β-globin gene **(Hu, Yin et al. 2017)** with consequently

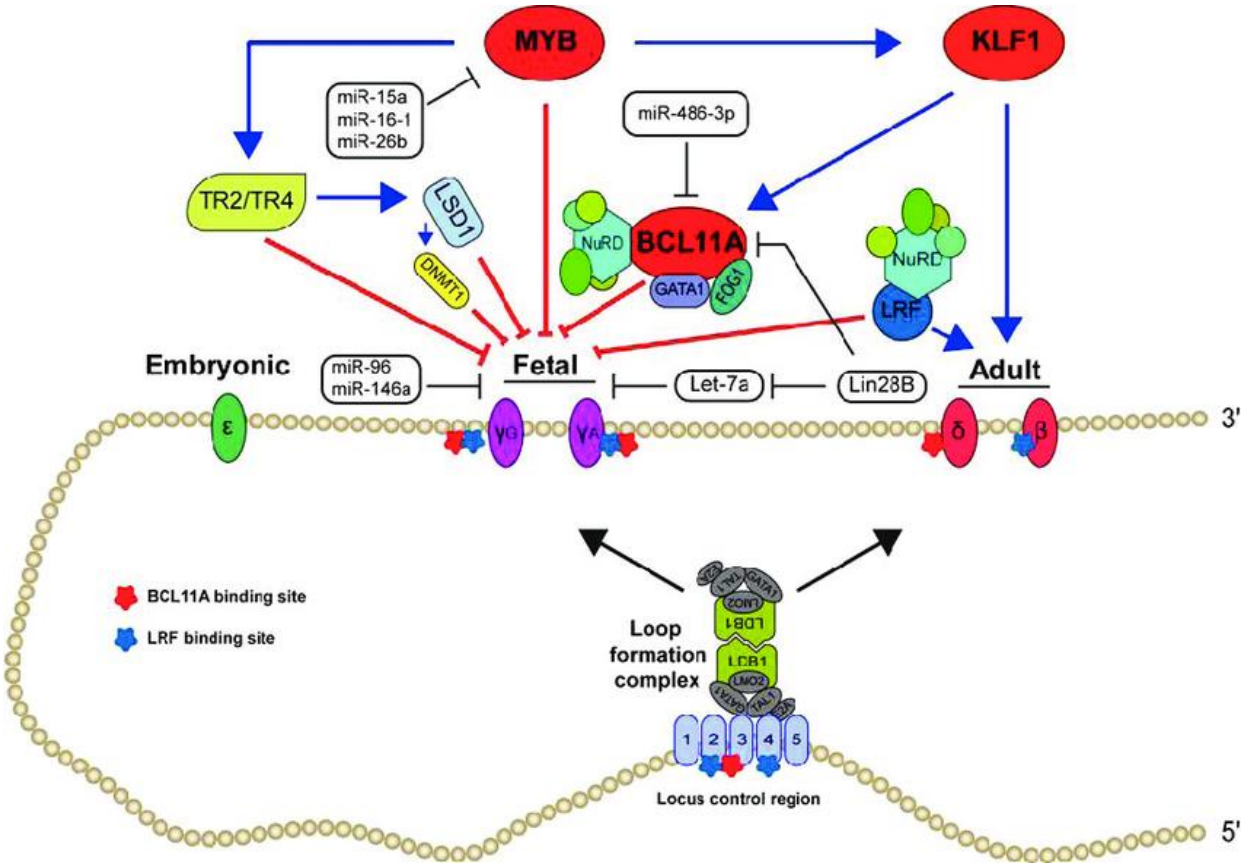
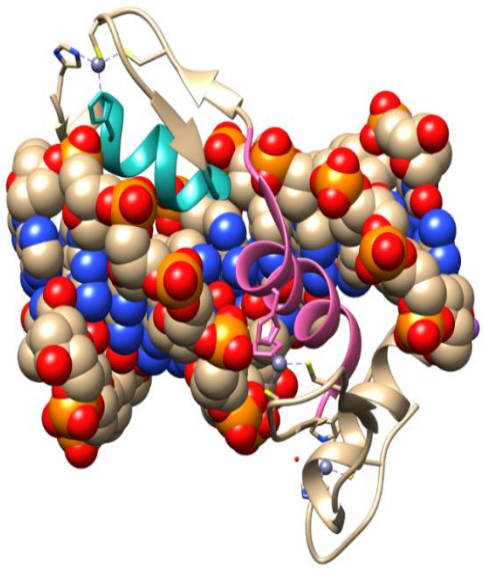


Figure 5.1: Partial gene regulatory network of γ-globin genes as obtained from multiple sources of evidence reproduced from* (Paikari and Sheehan 2018) *Activating regulation is shown by a pointed arrow and negative regulation is shown by a blunt arrow

down-regulation of the ϒ-globin gene and upregulation of the β-globin gene. In a second mode, BCL11A interacts with the NuRD complex by using a short peptide sequence in its N-terminal part causing repression or silencing of the gene **(Moody, Lo et al. 2018)**. Another approach can be using DNA-binding agents which target a specific sequence on the DNA, usually a protein binding site. Polyamides are best known for these kinds of molecules but so far have failed to

move to clinical trial due to a lack of adequate specificity **(Kawamoto, Sasaki et al. 2015, Kawamoto, Sasaki et al. 2016, Erwin, Grieshop et al. 2017)**. Conformationally constrained peptides are a new class of molecules that are capable of targeting specific DNA sequences directly. They are relatively easy to synthesize and appears to have enough specificity to selectively target specific DNA sequence in the cell; however their full selectivity needs to be fully assessed **(Mazumder, Maiti et al. 2012, Chakraborty and Roy 2016)**. A typical workflow for synthesizing such peptides from a known protein-DNA interface is described in Chakraborty and Roy **(Chakraborty and Roy 2016)**. These peptides and even their combination can be delivered systemically. Furnished with CPP motifs and nuclear localization signals, they reach their target cell affecting gene expression via binding to a specific DNA sequence.

Mutations in the regulatory sequences of the ϒ-globin gene disrupt the binding of the repressor proteins BCL11A and LFR (also known as ZBTB7A). Such gene editing may be one of the ways of genetic intervention **(Traxler, Yao et al. 2016)**. This type of mutation was observed in patients who downregulate their ϒ-globin expression in adulthood and therefore lead to an asymptomatic lifestyle despite loss-of-function mutations in the β-globin gene. It was recently shown that this mutation affects the binding of BCL11A and additional mutations in the DNA



gene

Figure 5.2: crystal structure of KLF4 complexed to its target DNA. DNA-interacting helices are in pink and cyan colours.

region led to similar consequences **(Martyn, Wienert et al. 2018)**. Thus, the possible therapeutic route that might be thought of is mutating the ϒ-globin promoter sequence in autologous hematopoietic stem cells.

5.1. Design of a peptide targeting KLF1 binding sites

The transcriptional regulator KLF1 is thought to play a key role in erythropoiesis by implementing the switch from fetal to adult hemoglobin **(Zhou, Liu et al. 2010)**. Thus, disruption of KLF1-mediated activation of these genes, reduction of KLF1 expression, and a combination of these steps might lead in turn to reduced activity or that of the aforementioned repressors LRF and BCL11A. While the crystal structure of KLF1 complexed to its target DNA has not been measured yet there exists a structure of KLF4 Zn finger domain bound to DNA (Fig.5.2) **(Schuetz, Nana et al. 2011)**.

. The DNA binding motifs of KLF1 and KLF4 share 90% homology (Fig.5.3) and this structure will form the basis for the design of the KLF-1 mimicking peptide. Simple binding of such a peptide to the KLF1 binding motifs in LRF and BCL11A promoter is likely to compete with the endogenous KLF1 binding. KLF1 binding sites to proximal LRF/ZBTB7A promoter have been mapped **(Norton, Funnell et al. 2017)** and those sites is used for targeting. Following the above-mentioned strategy, the constrained peptide that mimic KLF1 binding to its target was

designed. Based on KLF4 structure the DNA interacting helices were connected with a suitable linker and stabilized and are stabilized through incorporation of α-aminoisobutyric acid (Aib) as described **(Banerjee, Basu et al. 2002, Mazumder, Maiti et al. 2012).** The interacting residues are found to be from 321 to 336 and then from 348 to 357. The residues from 337 to 347 are found to be either not interacting or least interacting and

```
klf1      -TAEDPGVIAETAPSKRGRRSWARKRQAA HTCAHPGCGKSYTKSSHLKAHLRTH TGEKPY     309
klf4      ELMPPGSCMPEEPKPKRGRRSWPRKRTAT HTCDYAGCGKTYTKSSHLKAHLRTH TGEKPY     460
                 . : *    ******* *** *:*** : ****:********************

klf1      ACTWEGCGWRFARSDELTRHYRKH TGQRP FRCQLCPRAFSRSDHLALHMKRH L  362
klf4      HCDWDGCGWKFARSDELTRHYRKH TGHRP FQCQKCDRAFSRSDHLALHMKRH F  513
           * *:****:****************:***:** * ****************:
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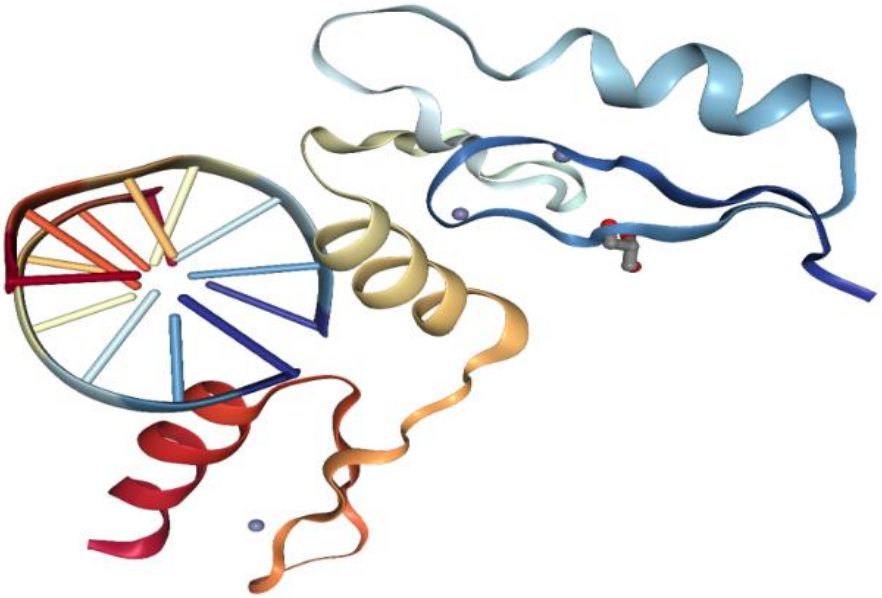


Figure. 5.3: The structure of Klf4-DNA binding complex based on Schuetz et al (Schuetz, Nana et al. 2011), pdb-created image) and the sequence alignment of the DNA binding domains of Klf1 and Klf4 done in blast2 sequence (upper panel).

therefore, these residues are deleted and the two interacting sequences are linked through a linker containing two glycine residues covering the distance (Figure 5.4). The residues 326L, 330Y, 333H, 353L and 356H which are highlighted by blue color in the figure 5.5 is least interacting and therefore were substituted by Aib and are shown in the figures 5.5 and 5.6.

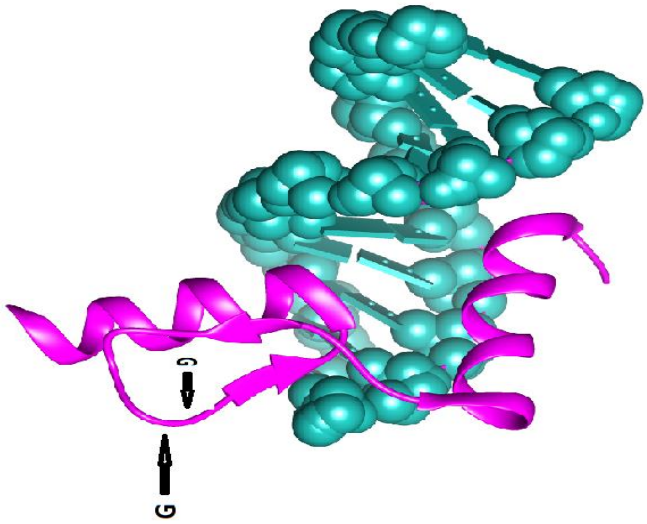
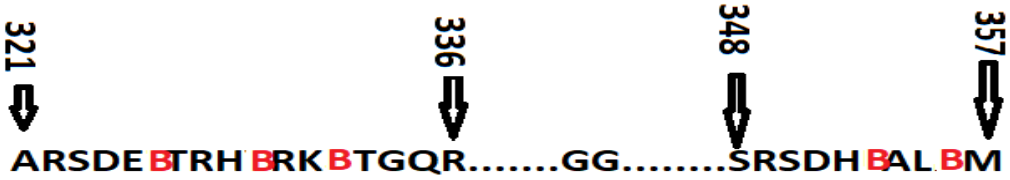


Figure. 5.4: A model og the structure of DNA bound with KLF1 peptide

Figure 5.5: Amino acid sequence of the unsubstituted KLF1 peptide.

Figu



5.2. Methods

The peptide was synthesized using MBHA resin in 0.1 millimole scale. The requisite amount of resin was weighed and swirled in DMF for half of an hour and then substitution of the resin was fixed using the first amino acid residue in exact calculated amount. Then subsequent amino acid residues were attached using five-fold molar excess of amino acid with respect to the one attached to the resin. When the peptide synthesis was complete the peptide was washed with dry DMF repeatedly. The peptide was then also dried by passing N_2. When the peptide synthesis was complete the peptide was washed with dry DMF repeatedly and finally dried by passing N2 gas for few minutes. The peptide was then labelled with 5(6)-Carboxyflourescein by incubating with 1:20 peptide and dye molar ratio using HOBT as the coupling agent for 4 hours at 250C. After incubation the peptide was washed with 20%

piperidine in DMF until the color of the washing becomes colorless and then dried with the help of nitrogen gas. The peptide was then detached from the resin using the mentioned protocol and washed repeatedly with chilled diethyl ether and finally dried. Now the peptide is ready for purification by HPLC. In HPLC a normal gradient is set up to 60% acetonitrile and the retention time of the peptide was 19-20 minutes.

5.3. Results and Discussion

In order to design a peptidomimetic which can be used to assay KLF1-DNA interaction, a conformationally-constrained helix of 29-mer derived from the KLF1 protein was designed whose sequence is as follows with a fluorescent label at the N-terminus:

FL-H_2N-ARSDEBTRHBRKBTGQRGGSRSDHBALBM-CONH$_2$

Figure 5.7: Sequence of the peptidomimetics with N-terminal fluorescent probe. FL stands for fluorescence tag 5(6)-Carboxyflourescein. B stands for Aminoisobutyricacid (Aib)

Figure 5.8. represents the HPLC chromatogram of the peptide at 215 nm and figure 5.9 represents the HPLC chromatogram of the peptide at 490 nm.

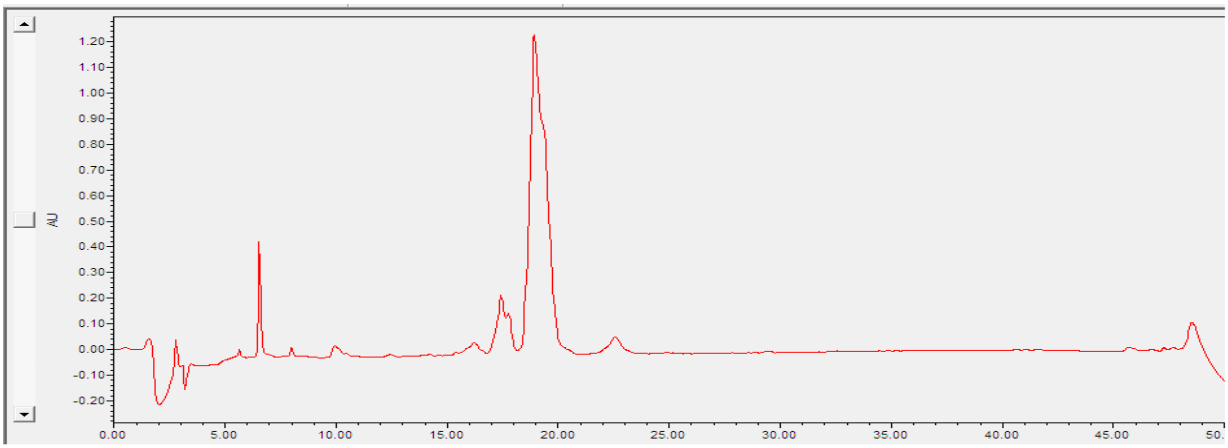


Figure 5.8: HPLC chromatogram of the peptide at 215nm.

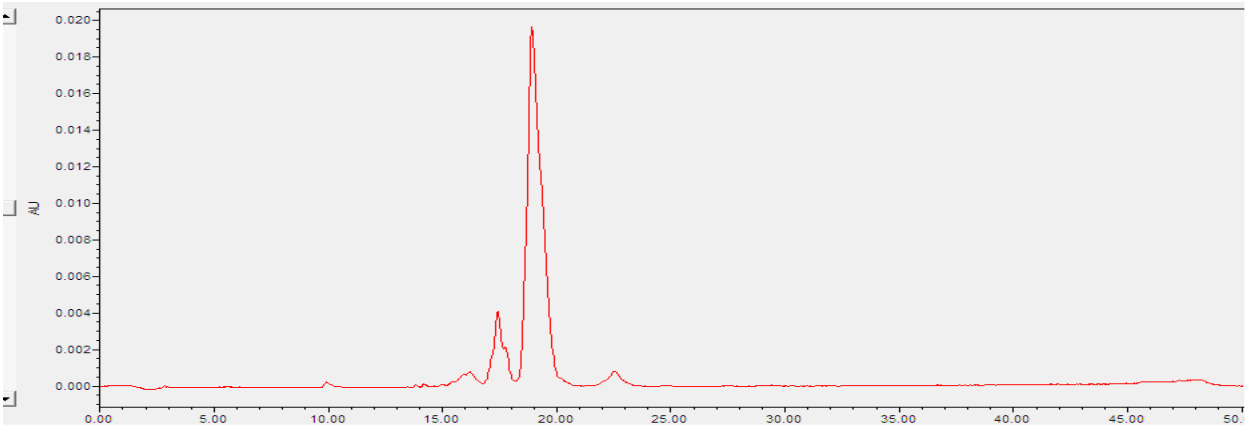


Figure 5. 9: HPLC chromatogram of the peptide at 490nm.

After the peptide was purified by HPLC its mass was analyzed by Electron spray ionization method which confirmed that peptide was correctly synthesized. Table 5.1 shows the mass of the different peptides. The ESI mass spectra of the peptides are given in figure 5.10, the ESI mass spectra of the unlabeled peptide. Figure 5.11, ESI mass spectra of labelled peptide.

Table 5.1: List of peptides with corresponding mass value.

No.	Peptide	Mass	Expected

		(Da)	Mass
1.	Unlabelled peptide:H_2N-ARSDEBTRHBRKBTGQRGGSRSDHBALBM-$CONH_2$	3159	3136
2.	Labelled peptide: FL-H_2N-ARSDEBTRHBRKBTGQRGGSRSDHBALBM-$CONH_2$	3491	3492

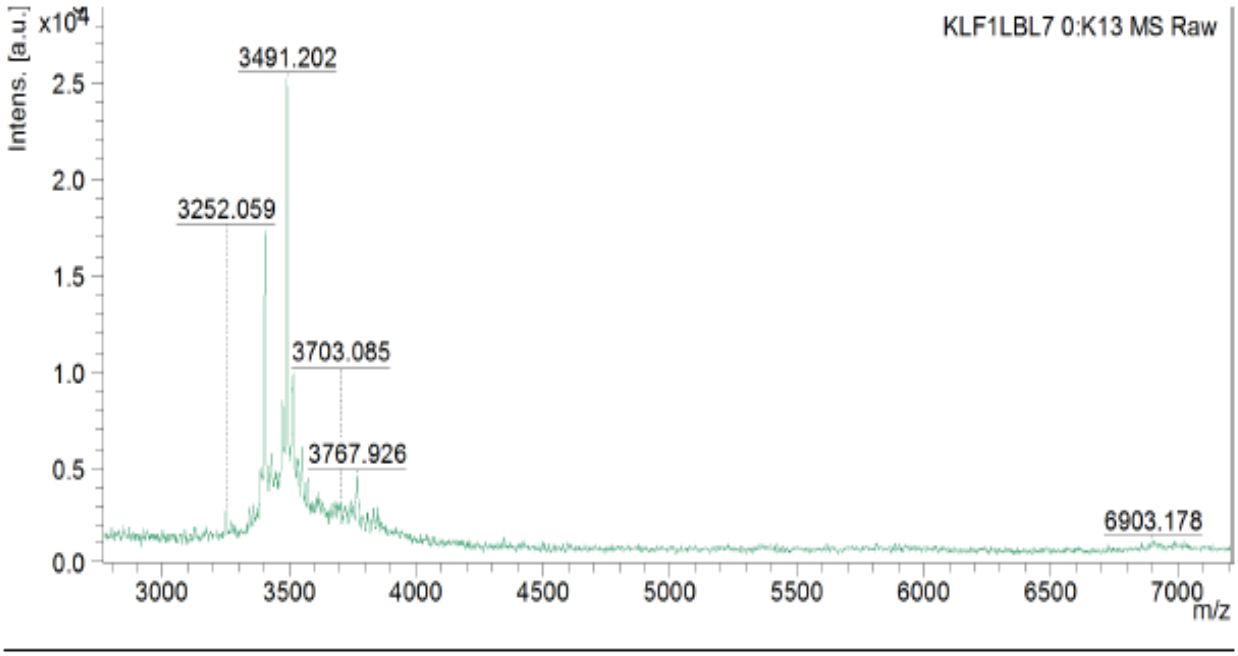


Figure 5.10: ESI mass spectra of labelled peptide.

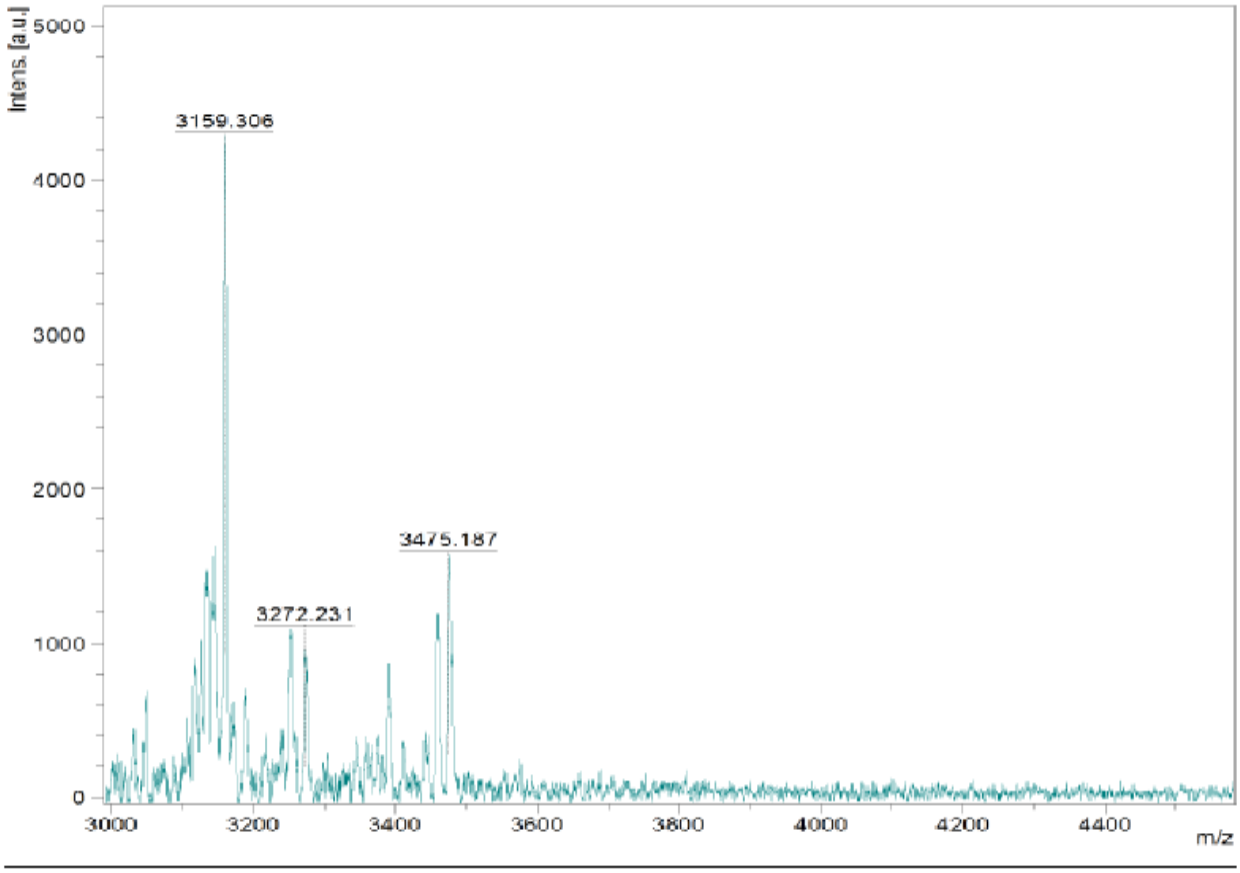


Figure 5.11: ESI mass spectra of unlabelled peptide.

In order to determine the interaction of the peptide with that of the designed DNA the titration of the peptide with DNA was performed by taking 1 µM concentration of the peptide with varying concentrations of DNA and it was found that the peptide interact with the DNA with a dissociation constant (K_D) value of 276±11.2 nM (Figure 5.12).

In order to determine the specificity of the KLF1 peptide with the specified DNA, a random DNA was selected and its binding isotherm with the random DNA was studied by sane method and no detectable change was observed with random DNA, indicating that the peptide has considerable degree of specificity for the target DNA.

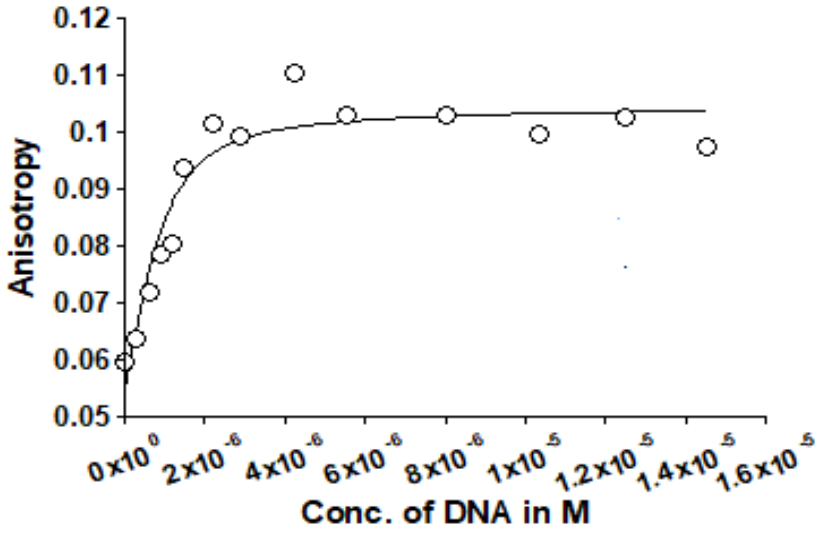


Figure 5.12: Average of triplicate data of binding isotherm of the KLF1 labelled with 5-(6)-carboxy flourescein peptide with DNA.

In summary, we have designed a novel linker between two helices of a zinc finger domain to produce a peptide that binds to the target DNA sequence with high affinity and considerable degree of specificity. To our knowledge, this is the first example of a modified peptide that mimics DNA binding of a zinc finger domain. Given the preponderance of Zinc finger-containing transcription factors in eukaryotes, these types of peptides, may be useful for investigating the biological effects of such transcription factors and may offer an avenue for peptide therapeutics, specifically targeting transcription factors.

CHAPTER 6

BERBERINE AND ITS CONJUGATION WITH A CYCLIC PEPTIDE

Peptide-drug conjugates are drawing increasing attention as a new class of therapeutics. Peptides can act as a carrier to direct drugs to specific tissues or it can be used to increase the specificity of the target binding. in this chapter I explore a way to prepare a conjugate of a very common natural product Berberine, which shows many therapeutic effects. Barberry (Berberis vulgaris L.) is a well-known medicinal plant which contains Berberine. Berberine is also found in some other plants. It is also used as a food additive (KHOSROUKHAVAR, Ahmadiani et al. 2010). Berberine is an isoquinoline alkaloid that belongs to the structural class of protoberberines and is present in many plants including B. Vulgaris (Imenshahidi and Hosseinzadeh 2016).

Berberine (BBR) has been a part of Chinese and Ayurvedic medicine for thousands of years. It works in multiple different ways and is able to make changes in cells. BBR has a molecular weight (MW) of 336.37 Da and can be easily obtained from plants or through de novo synthesis **(Kong, Wei et al. 2004, Huang, Zeng et al. 2011)**. It has anti-bacterial properties **(Xie, Johnson et al. 2012)** and owing to its excellent safety profiles in humans, BBR has been utilized for many decades in China as an over-the-counter medicine for bacterial diarrhea. Recent studies have indicated that BBR may be effective in treating chronic, multifactorial diseases, including diabetes, hyperlipidemia, heart diseases, cancers, and inflammatory diseases. In addition, laboratory studies have identified several molecules and signaling pathways that account for its therapeutic effects. Importantly, the clinical effects of BBR are due to multiple target molecules and/or mechanisms. Treatment of multifactorial chronic diseases with agents that regulate multiple molecular targets may be particularly effective **(Yao, Kong et al. 2015)**.

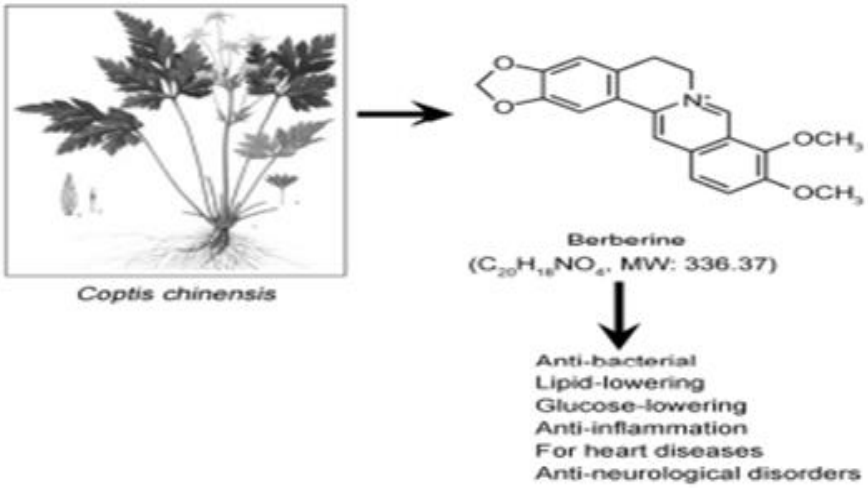


Figure 6.1. Chemical structure of berberine, a botanic compound with multiple effects against chronic diseases in humans (Google image).

As a model I have chosen the peptide described in a previous chapter that was derived from the human La protein that has been studied extensively and has shown very significant RNA binding and specific antiviral effect against Hepatitis C virus. The linear peptide has a core sequence of K Y K E T D L. Linear peptides are generally degraded by cellular and serum proteases and cyclic peptides generally resist such degradation. In order to prepare a drug conjugate that may survive intracellular and serum proteases better, I have cyclized the 4 N-terminal amino acids. Preparation procedure and characterization will be described in subsequent sections.

6.1. Materials and methods

6.1.1. Materials

6.1.1.1. Chemicals and Reagents

Fmoc-amino acids, Rink amide MBHA resin, 2-(1H-benzotriazole-1-yl)-1, 1, 3, 3-tetramethylaminiumtetrafluoroborate (TBTU), hydroxybenzotriazole (HOBt) and N, N, N', N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) were purchased from Novabiochem. N, N, -diisopropylethylamine (DIPEA), thioanisole, 1, 2-ethanedithiol (EDT), trifluoroacetic acid (TFA), ammonium iodide (NH_4I), dimethyl sulfide (Me_2S) and HPLC grade acetonitrile were purchased from E-Merck, Germany. Triisopropylsilan (TIS) was purchased from Sigma Chemical Company (St. Louis, MO, USA). N, N-dimethylformamide (DMF), diethyl ether (Et_2O), piperidine and HPLC grade water were procured from Spectrochem. Reverse-phase Hypersil Gold C-18 HPLC column was acquired from Thermo Corporation Limited. Cuvettes for spectroscopic analysis were purchased from Hellma Analytics. Berberine is purchased from Pierce (Germany) and iodoacetic acid is purchased from Across (USA). All the solvents used such as methanol, ethanol, chloroform and dichloromethane were purchased from Merck (India).

6.1.1.2. Peptide synthesis and purification

All peptides were synthesized based on 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with a capping step consisting of 5% acetic anhydride and 5% lutidine in DMF after each coupling on Rink Amide PEGA resin using a peptide synthesizer (Protein Technologies). Fmoc-

amino acids were activated with TBTU in the presence of HOBt and DIPEA. Peptides were cleaved from the resin and side-chain protecting groups were removed by incubating with 94% TFA, 2.5% EDT, 1.5% Thioanisole, 1.5% water, 0.5% TIS for 3hours at 25 °C. The crude peptides were purified by HPLC on a reverse phase Hypersil Gold C_{18} column using 80% Acetonitrile in 0.01% TFA and characterized by Electron Spray Ionization mass spectrometry (ESI-MS) and Matrix assisted laser desorption ionization-Time of flight (MALDI-TOF).

6.3. Results and Discussion

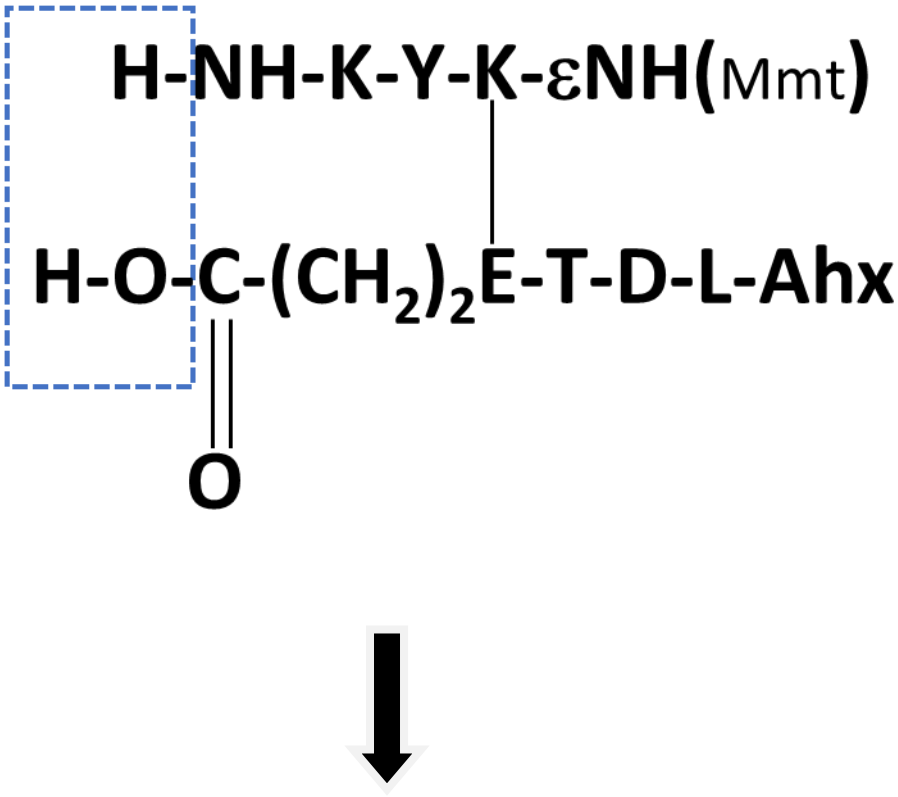
Internal ribosomal entry sites (IRES) mediated translation of hepatitis C virus has been implicated by human La protein. It has been demonstrated by Mondal et. al earlier that the RNA recognition motif (RRM) including the residues 112 to 184 of La protein interacts with the HCV IRES near the initiator AUG codon **(Mondal, Ray et al. 2008)**. Therefore, a synthetic peptide 24 mer was designed and synthesized by Roy and co-workers that retains RNA binding ability and competes with La protein binding to the HCV IRES and inhibits translation. The action of the peptide is supposed to interrupt the assembly of 48S complexes and is found to be accumulated in the preinitiation complexes that are found to be incompetent for the 60S ribosomal subunit joining. NMR spectroscopy revealed the putative contact points that have been mutated exhibiting reduced RNA binding and translational inhibitory activity. The residues that are responsible for RNA binding were found to form a turn in the RRM (112-184) structure. A 7-mer peptide comprising this turn shows a significant effect in translational inhibitory activity. Therefore, a better conformationally mimetic peptide containing beta turn is suggested and confirmed by NOE experiments. In order to design a cyclic peptidomimetic that can

function as a peptide-based inhibitor of HCV RNA virus, a conformationally constrained cyclic peptidomimetic of octamer including one linker AHX was synthesized. The sequence of the linear peptide is shown in figure 6.2 as follows **(Manna, Kumar et al. 2013).**

Figure 6.2: Sequence of the linear peptide

First, this peptide was synthesized using Rink amide PEGA resin in 0.1 mmole scale. For the first coupling, the substitution of the resin was fixed by using the procedure mentioned in chapter 1. After that the successive amino acids are linked using six times molar excess of the amino acid with respect to the resin-bound one, using TBTU as the activating agent and DIPEA as a base. The lysine marked as red used is simply Fmoc-Lys(boc)-OH whereas the lysine marked as green was orthogonally protected i.e., Fmoc-Lys (Mmt)-OH. The cyclization of the peptide was done using the α-NH2- group of the N-terminal lysine marked as red with the γ-COOH- group of the glutamic acid marked as blue using PYBOP as the coupling agent along with HOBT. Both coupling agents were taken 20-fold molar excess with respect to the resin-bound amino acid and the coupling was continued for 18 hours to ensure the cyclization of the peptide. The cyclization is shown in figure 6.3.

The orthogonal protection of fourth lysine is the cleaved using 0.1% acetic acid in dichloromethane. The same procedure of cleavage was repeated thrice to ensure the maximum extent of cleavage. In the ε-NH_2- group of fourth lysine an Ahx through its carboxyl group was coupled to keep the chemical analogue of berberine at a distance from the peptidomimetic sequence as shown in the figure 6.4 below.


H-NH-K-Y-K-εNH(Mmt)
H-O-C-(CH2)2E-T-D-L-Ahx
O

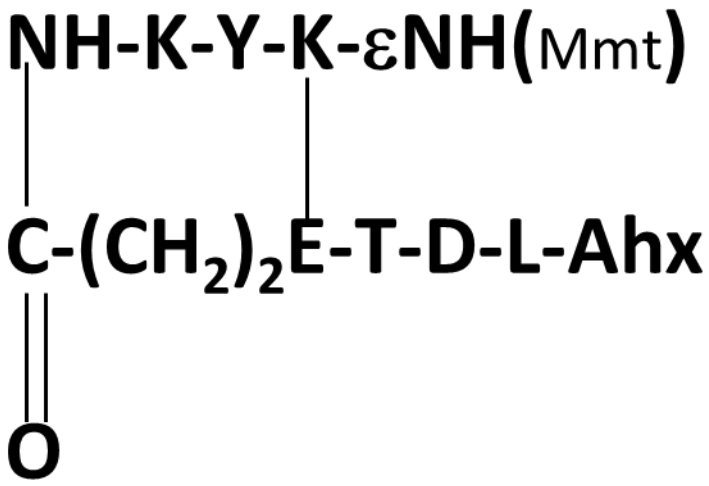


Figure 6.3: Cyclization of the four N-terminal amino acids.

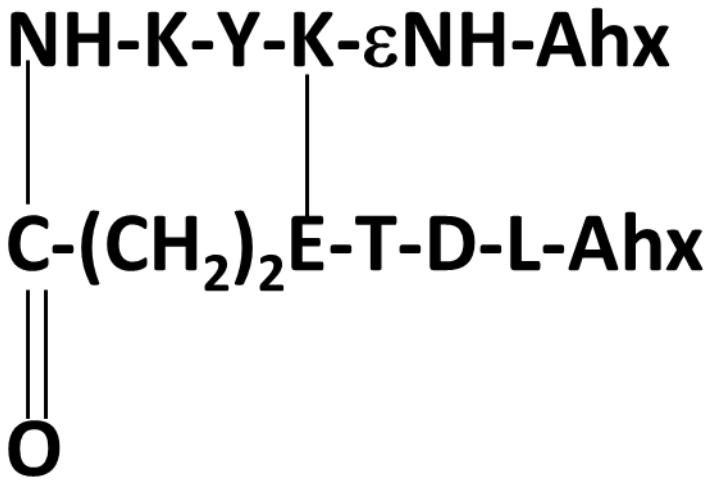


Figure 6.4: The sequence of the cyclic peptide with two Ahx moieties. The Ahx connected to the ε-NH has a NH2 group.

After the synthesis was completed, the peptide was cleaved from the resin by incubating with 94% TFA, 2.5% EDT, 1.5% Thioanisole, 1.5% water, 0.5% TIS for 3hours at 25 °C. The crude peptides were purified by HPLC on a reverse phase Hypersil Gold C_{18} column using 80% Acetonitrile in 0.085% TFA and water in 0.1% TFA. An isocratic gradient of 32% acetonitrile in 0.085% TFA and 68% of water in 0.1% TFA was run till 65 minutes and the pure peptide was obtained at near about 35 minutes. The HPLC chromatogram of the pure cyclic peptide is shown in the figure 6.5.

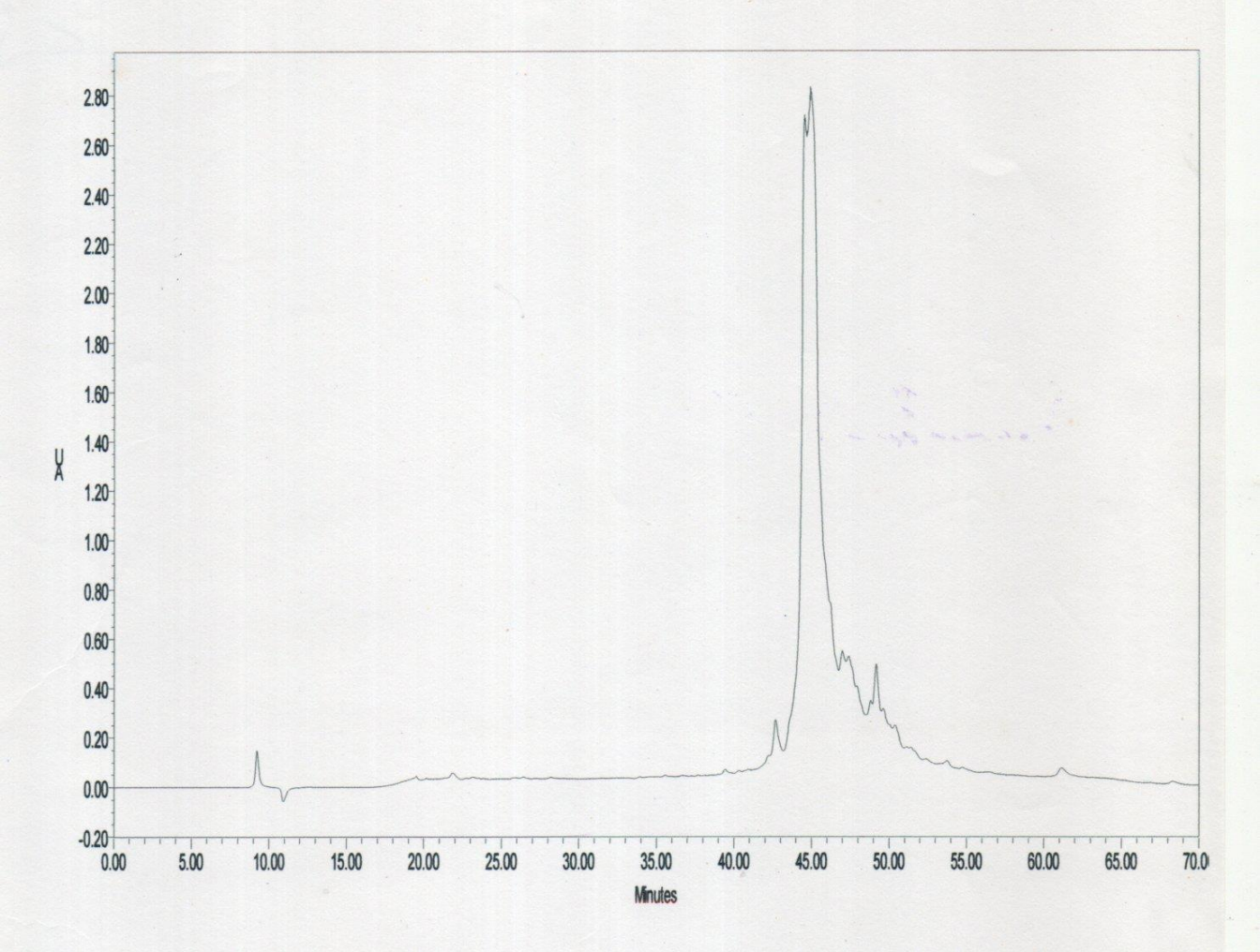


Figure 6.5: HPLC chromatogram of the cyclic peptide. In horizontal axis time in minute is recorded whereas in vertical axis AU ie absorption unit is recorded.

Table 1: Mass of the peptides.

Name of the peptides	Expected mass	Obtained mass
Mass of the linear peptide	1009 daltons	1010 daltons
Mass of the cyclic peptide	991 daltons	991 daltons

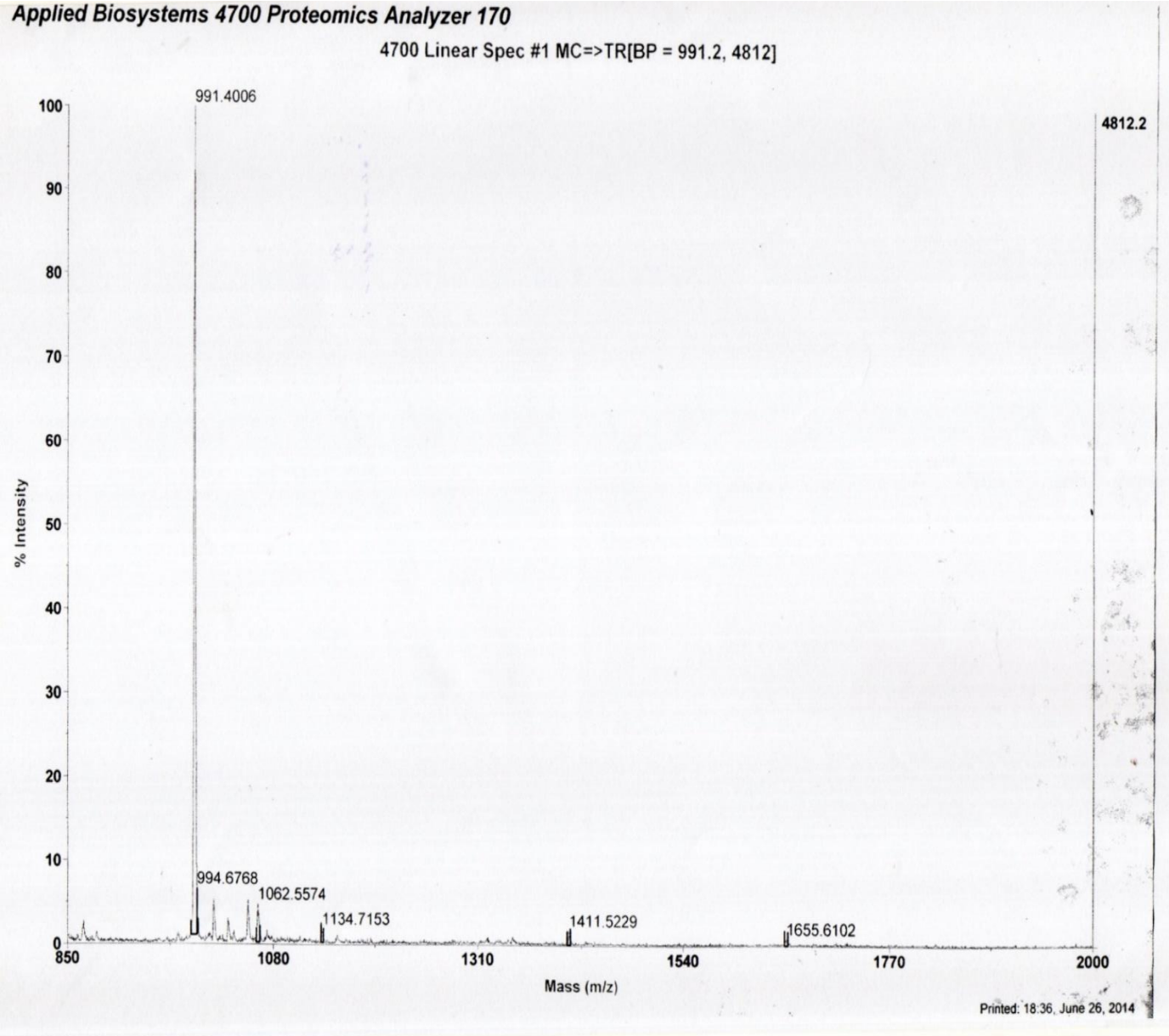


Figure 6.6: MALDI mass spectrum of the cyclic peptide

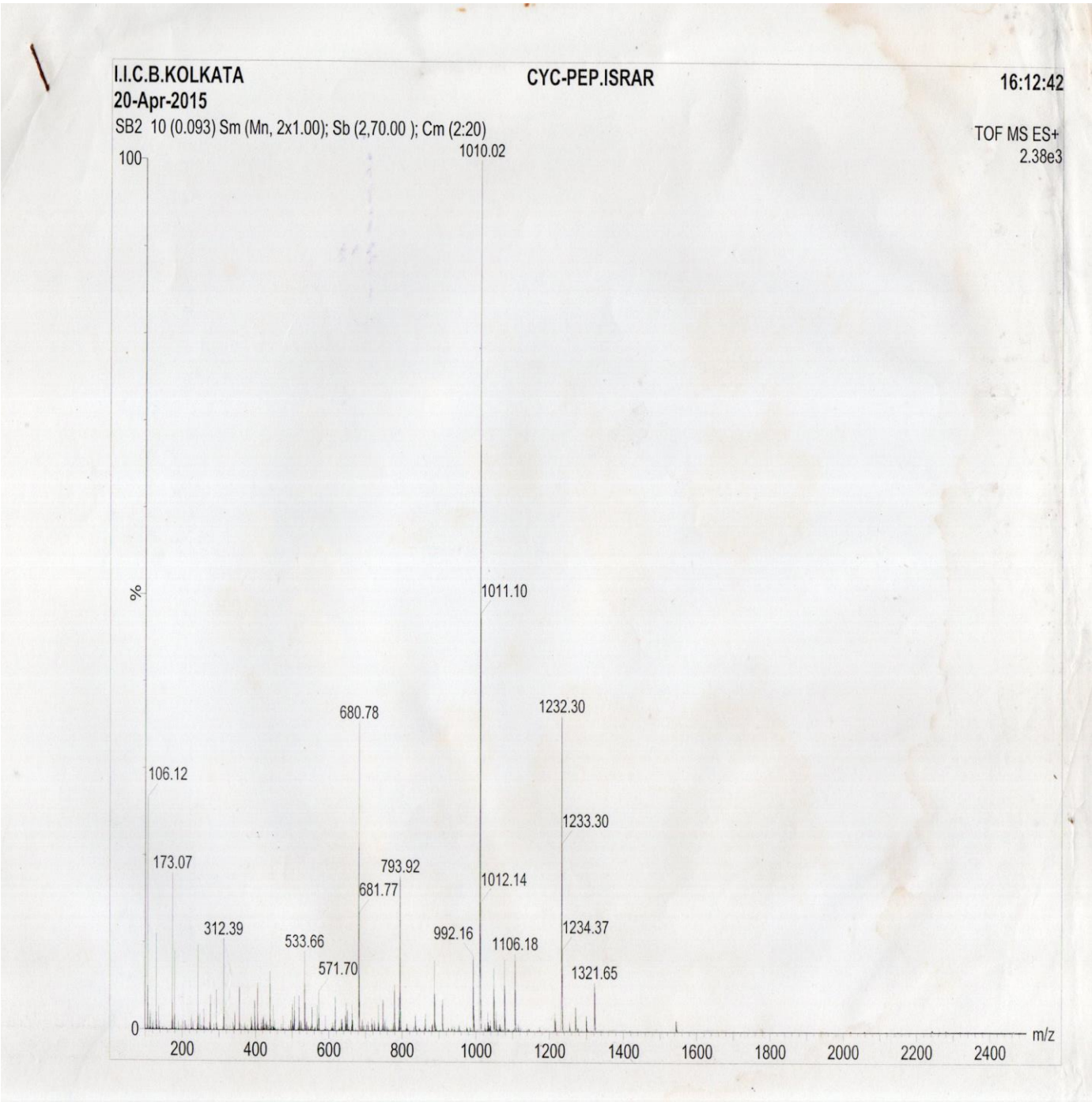
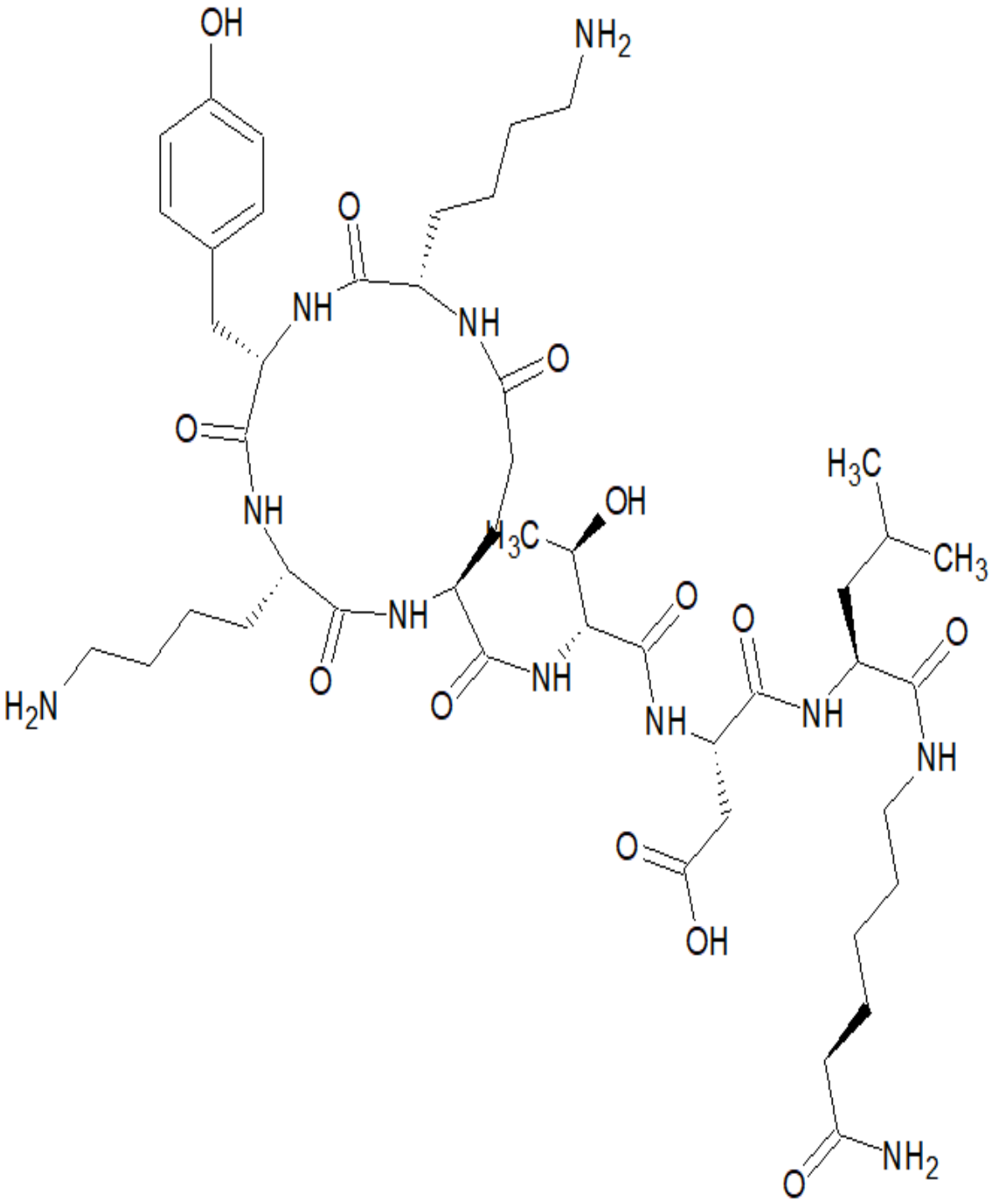


Figure 6.7: ESI Mass spectrum of the linear peptide.

Full atomic structures of the cyclic peptides are shown below.



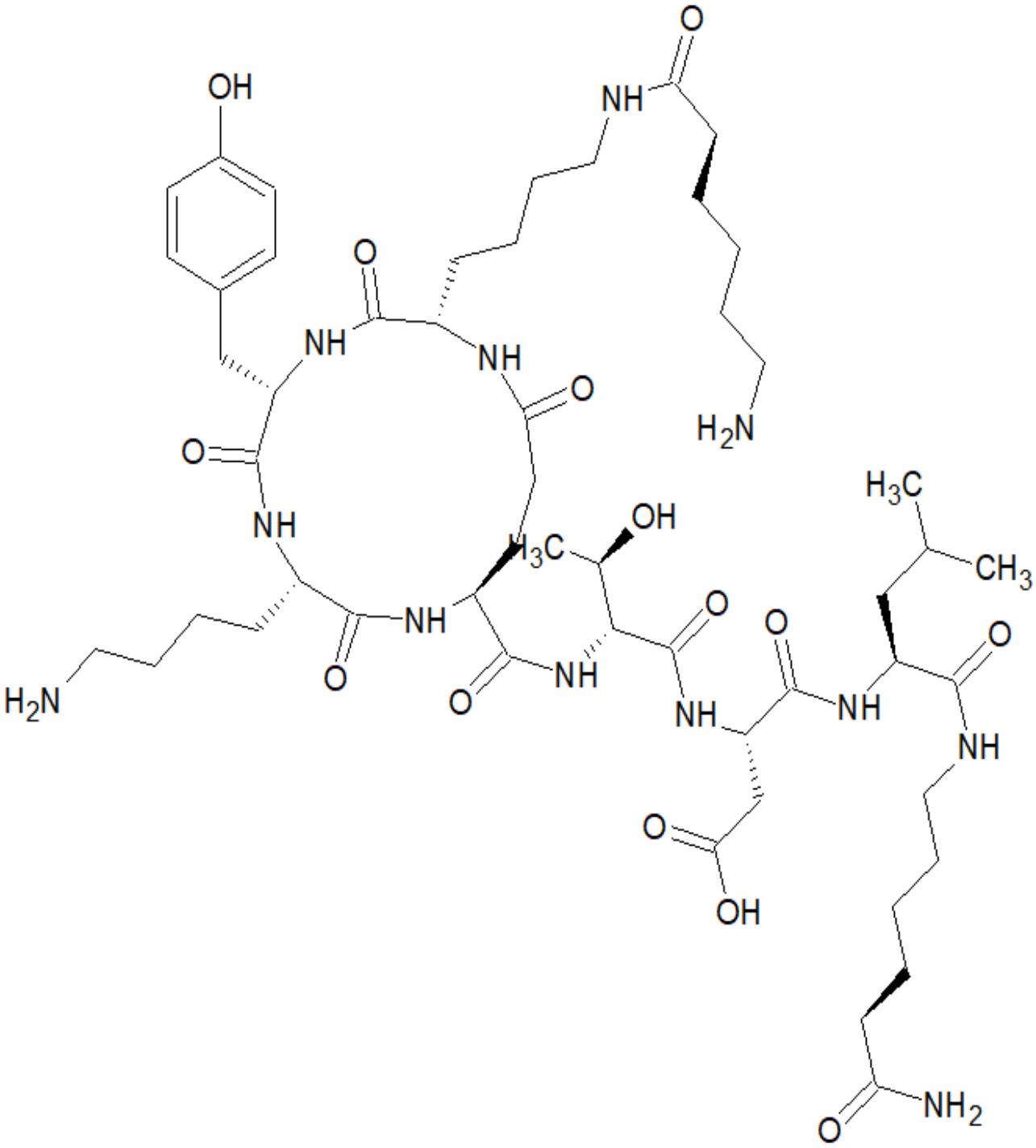


Figure 6.8. Atomic structures of the cyclic peptides. Upper panel with one Ahx, the lower panel with two Ahxs.

Synthesis of the berberine derivative

Synthesis of berberrubine:

M/Z = 336 M/Z = 322

500 mg of the commercially available berberine is taken in a 100 ml round bottomed flask and heated at 200^0C under vacuum and the pressure is maintained in between 25 to 30 atmosphere (Milata, Svedova et al. 2019) for half of an hour and TLC was observed as shown in the figure

6.9 below:

Figure 6.9: TLC of the reaction confirming the synthesis of berberrubine

The ESI mass spectrum of the berberrubine is shown in the figure 6.10 as follows:

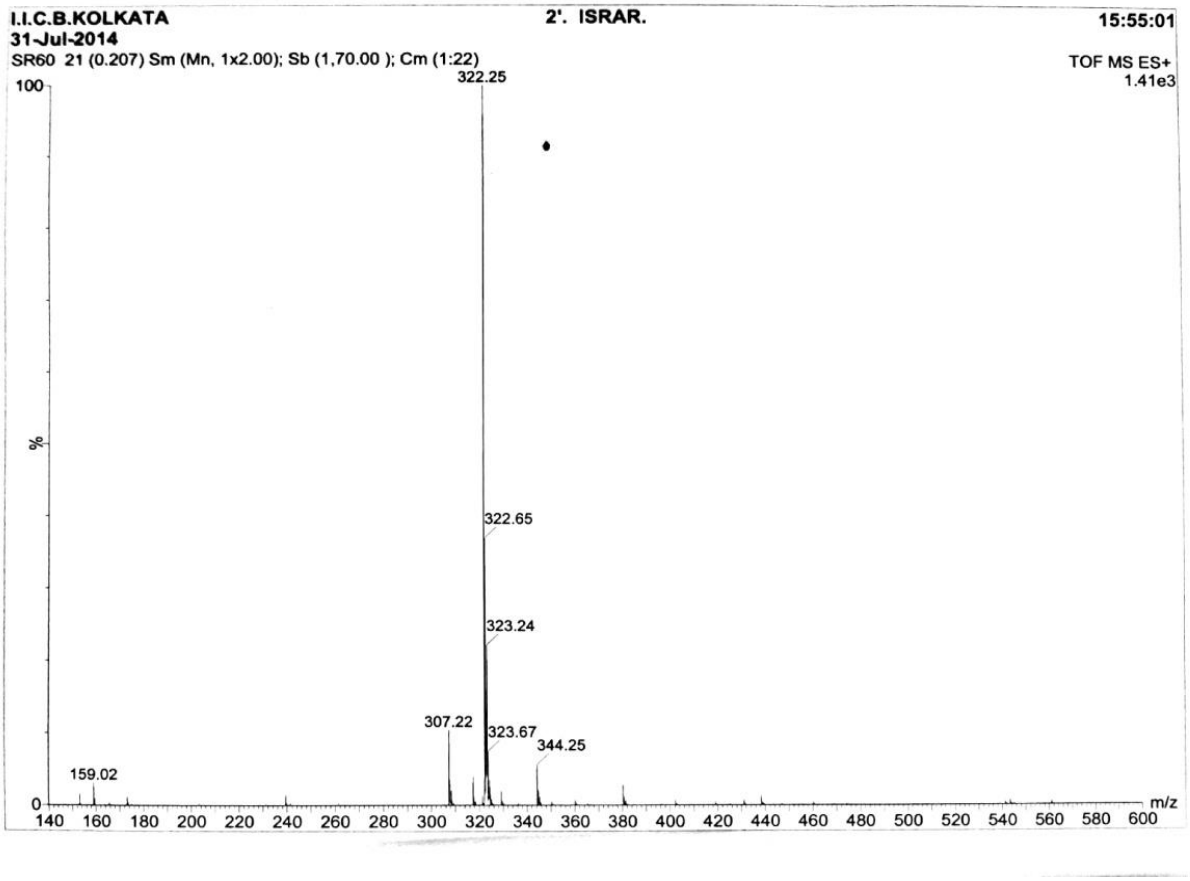
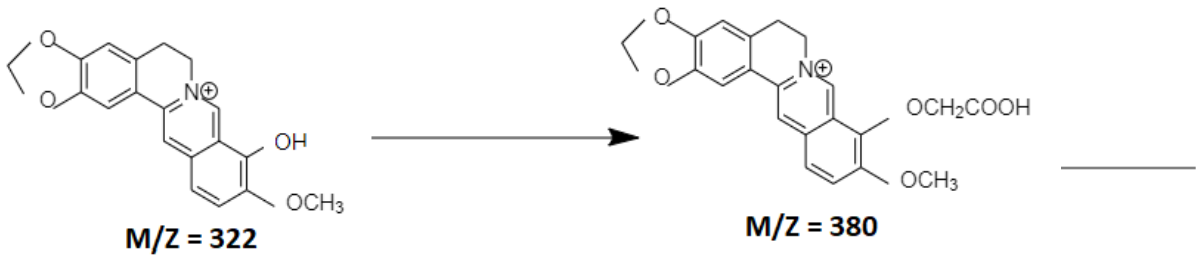


Fig: 6.10. ESI mass spectrum of Berberrubine.

Alkylation of berberrubine:



Demethoxy berberine which is alsoknown as berberrubine0.03726 mmole (12 mg approximately) was mixed with 10 times iodoacetic acid 0.3726 mmole (69.3mg approximately) and was dissolved in a mixture of solvent containing DMF, water and bicarbonate buffer pH= 9 in the ratio 3:2:5. The reaction was continued at 60^0C for 72 hours and then the product was extracted by using DCM and the TLC was performed in 10% methanol in DCM which is shown in the figure 6.10 as follows:

The ESI spectrum of the alkylated berberine derivative is shown in the figure 6.11. The product is dissolved in minimum volume of 100% methanol with heating and kept in dark and cold place for overnight for recrystallization. After recrystallization the product is dried.

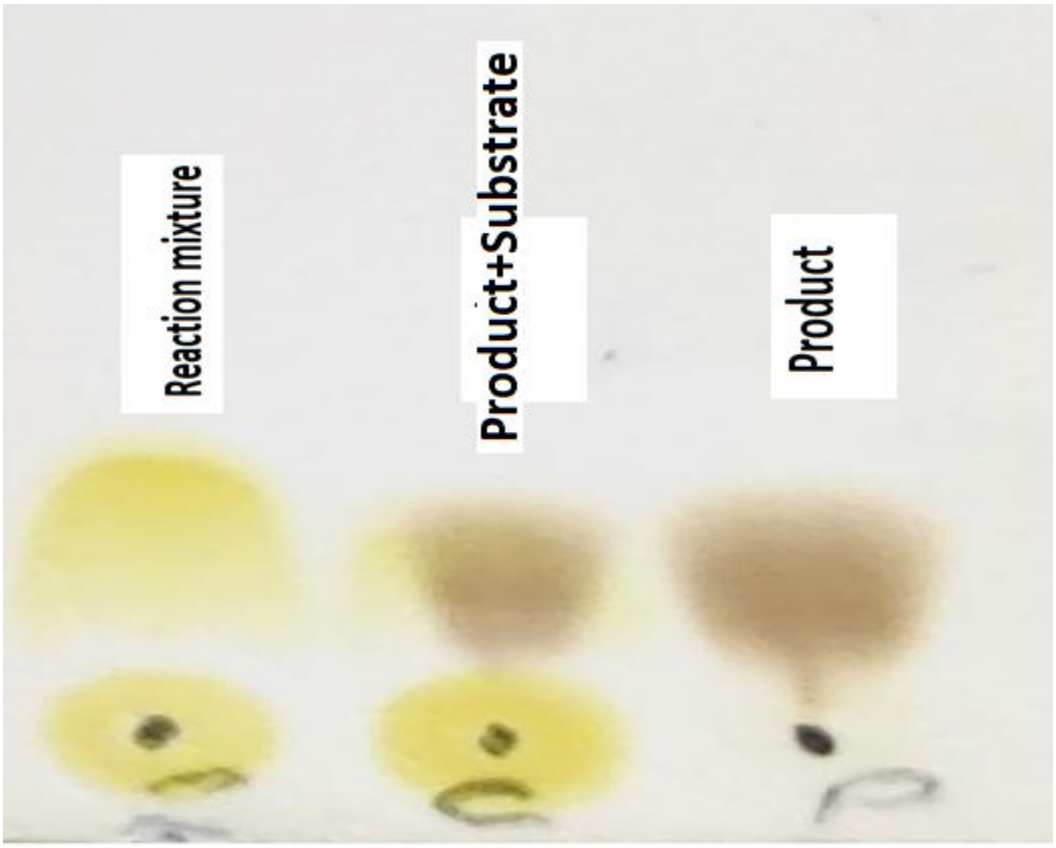


Figure 6.11: TLC of the reaction confirming the alkylation of demothoxy Berberine.

Finally, the alkylated berberine derivative was coupled with the cyclic peptide by solid phase method. 0.005 mmole resin of the cyclic peptide was mixed with 10 times of the bebrberine derivative using a mixture of pybop and HOBT as the coupling agent and DIPEA as a

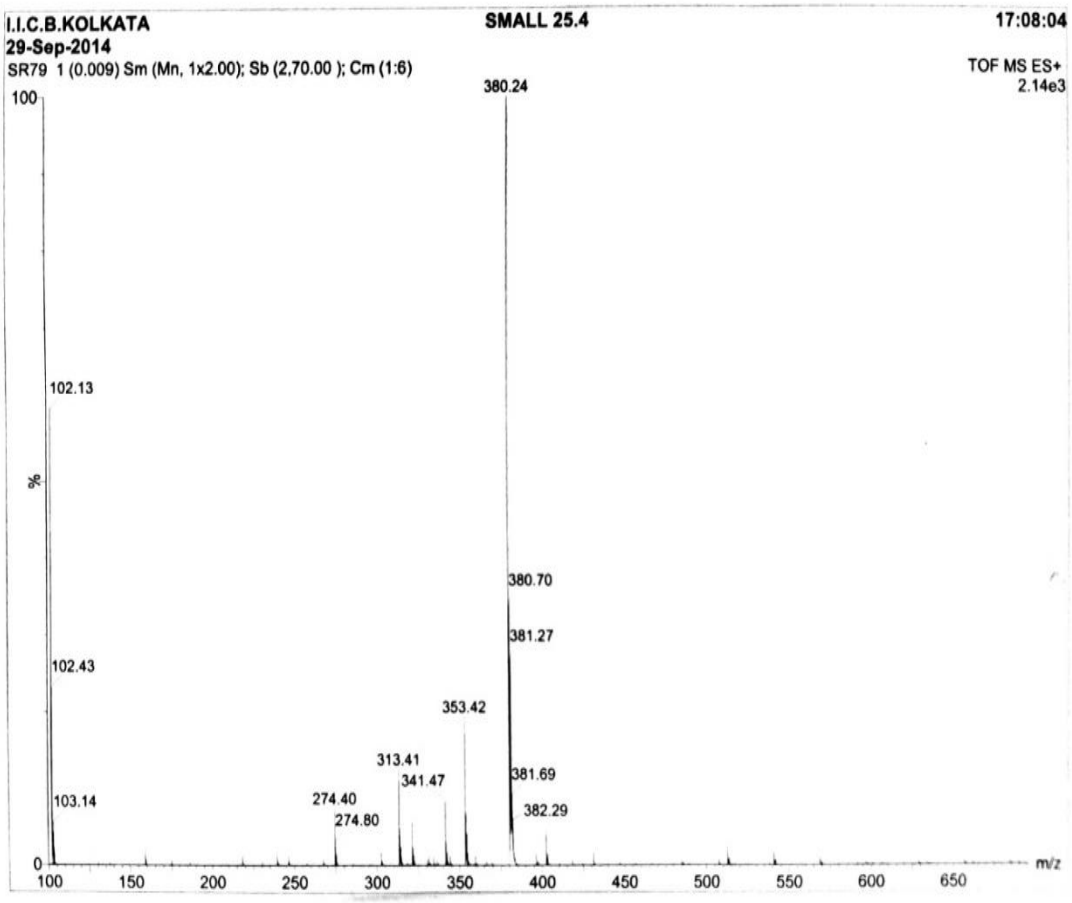


Figure 6.12: ESI chromatogram of alkylated berberine.

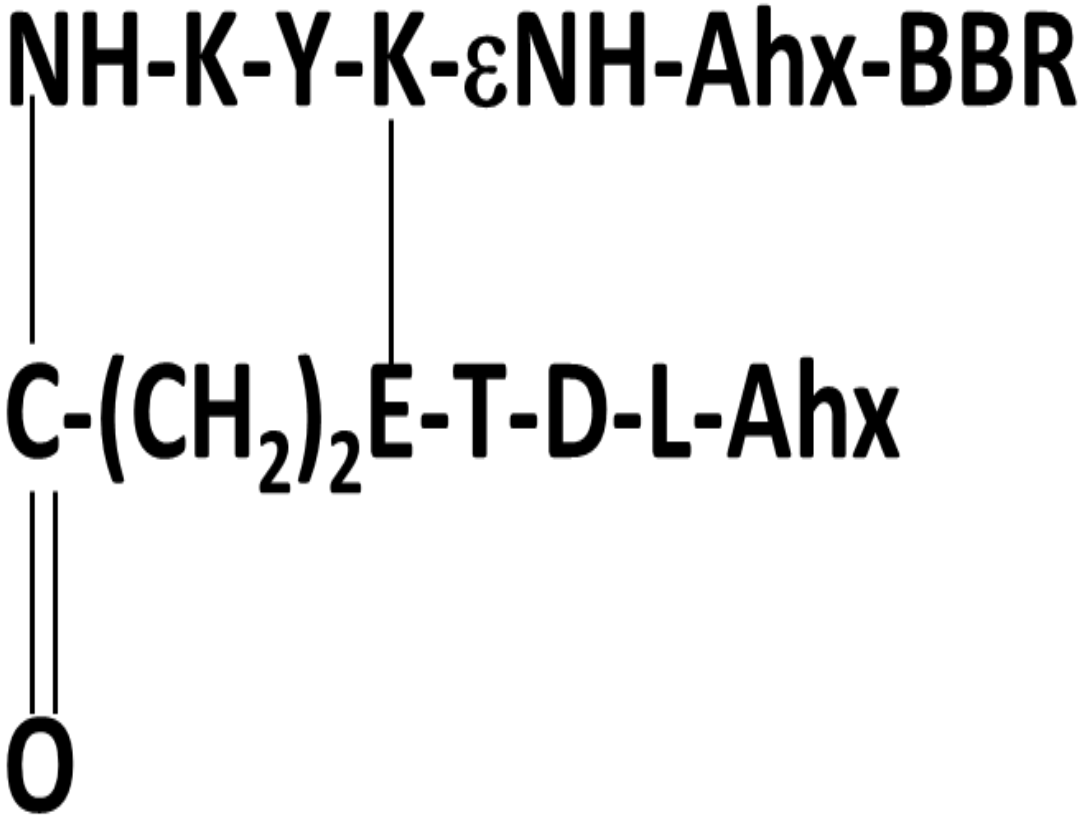


Figure 6.13: Complete structure of the Berberine-peptide conjugate.

base and whole night the coupling was continued under the inert atmosphere of nitrogen. Then the resin was cleaved using the same procedure as mentioned earlier in this chapter and the final product was recrystallized followed by repeatedly washing with chilled ether. The scheme of the product and the ESI chromatogram of the final product whose expected mass is 1465 daltons is seen in the figure 6.12 and 6.14, respectively.

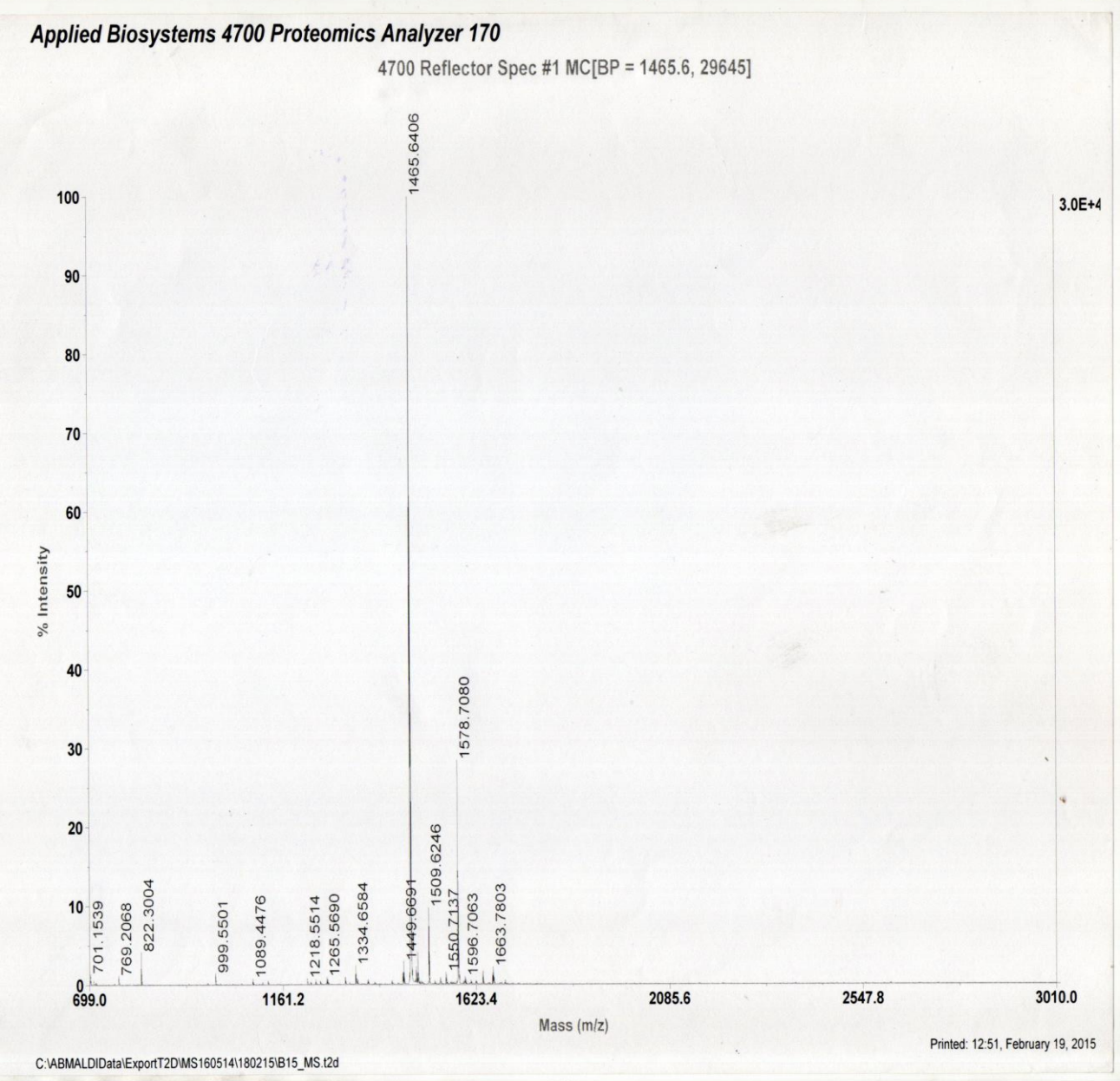


Figure 6.14: ESI mass spectra of cyclic peptide conjugated with berberine derivative.

In future, I plan to test the bioactivity of this berberine-peptide conjugate.

CHAPTER 7

NANOSCALE MODIFICATION OF BIDENTATE BRANCHED PEPTIDE

In previous chapters, I have shown how to design peptides that target specific proteins and develop them as candidate molecules. However, in real life, they need to be delivered inside a cell in live animals. I have also shown that when a peptide alone gets injected into the bloodstream, it has a tendency to clear relatively rapidly from the serum for various reasons. This requires higher doses to compensate for the rapid clearance. With the development of nanoparticles as vehicles for the delivery of various drugs into cells within an animal, it is natural to contemplate that peptides may be effectively delivered using nanoparticles or liposomal encapsulation. In this chapter, I have described how a peptide can be effectively conjugated to a glucose-based nanoparticle that is fully biocompatible and I have characterized the peptide-nanoparticle conjugate as well. We have chosen a model peptide for the purpose; in this case we chose the linear peptide, KYKETDLAhx, described in chapter 6.

Nanoparticles are material particles of very small dimensions whose diameter ranges from roughly 1 nm to 100 nm (Vert, Doi et al. 2012, Langley and Lipton 2013). The properties of nanoparticles differ markedly from those of the larger particles of the same material as the dimension of the majority of nanoparticles lies within a few atomic diameters of its surface. That is the reason for properties of the surface layer may dominate over the bulk of the material. The application of nanomaterials in the industrial field is commonly known as Nanotechnology which has been explored extensively, particularly in the research field since the last century. Currently, this technology is being used most extensively in various biomedical research particularly carbon-based single-walled or multiple-walled nanotubes are being increasingly used for the transport of therapeutic agents in drug delivery vesicles (Quan, Xie et al. 2011, Selvi, Chatterjee et al. 2012, Ali, Mukhtar et al. 2018).

Amorphous carbon nanospheres derived from glucose are non-toxic and intrinsically fluorescent which have the ability to deliver drug molecules directly inside the nucleus (Selvi, Jagadeesan et al. 2008). It is an intracellular carrier and the surface of these particles is specifically functionalized due to the richness of charge and does not require any other modifications; therefore, it targets the cells having more glucose transporters such as the glial cells. The intrinsic fluorescent property of this carbon nanosphere helps in detecting the cellular localization of these without any other fluorescent tags. The sphere is highly specific in its action and is found to be nuclear in localization in mammalian cells without any toxic effect. In addition to this, the foremost ability of these spheres is that they can cross the blood-brain barrier and be localized in the brain without getting localized in the liver and spleen and it has been observed that these spheres are being removed from these tissue over time (Selvi, Jagadeesan et al. 2008).

7.1. Materials and methods

7.1.1. Materials

7.1.1.1. Chemicals and Reagents

Fmoc-amino acids, Rink amide MBHA resin, 2-(1H-benzotriazole-1-yl)-1, 1, 3, 3-tetramethylaminiumtetrafluoroborate (TBTU), hydroxybenzotriazole (HOBt) and N, N, N', N'-tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU) were purchased from Novabiochem. N, N, -diisopropylethylamine (DIPEA), thioanisole, 1, 2-ethanedithiol (EDT), trifluoroacetic acid (TFA), ammonium iodide (NH4I), dimethyl sulfide (Me2S) and HPLC grade acetonitrile were purchased from E-Merck, Germany. Triisopropylsilan (TIS) was purchased from Sigma Chemical Company (St. Louis, MO, USA). N, N-dimethylformamide (DMF), diethyl

ether (Et2O), piperidine and HPLC grade water were procured from Spectrochem. Reverse-phase Hypersil Gold C-18 HPLC column was acquired from Thermo Corporation Limited. Cuvettes for spectroscopic analysis were purchased from Hellma Analytics. Berberine is purchased from Pierce (Germany) and iodoacetic acid is purchased from Across (USA). All the solvents used such as methanol, ethanol, chloroform and dichloromethane were purchased from Merck (India). CSP nanoparticles were obtained as a gift from Tapas Kr. Kundu's Laboratory. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) was purchased from sigma Aldrich and Sulfo-NHS (N-hydroxysulfosuccinimide) ester was purchased from Thermo Scientific.

7.1.1.2. Peptide synthesis and purification

All the peptides were synthesized on an Aaptec synthesizer at 0.2 mmole scale by using a solid phase peptide synthesis strategy using 9-fluorenylmethoxy carbonyl chemistry and Rink Amide resin. All the protected amino acids were purchased by Novabiochem. Aspartic acid, threonine, tyrosine and serine had tertiary butyl arginine had pentamethyl dihydrobenzofuran and lysine had tertiarybutoxycarbonyl protecting group at the amino group of β chain. N-terminal Fmoc protected amino acids were coupled in the COOH group using O-(Benzotriazole-1-yl)-N,N,N',N'-tetramethyl uronium tetrafouroborate(TBTU) as an activating agent with N-hydroxybenzotriazole(HOBT) and N,N-diisopropyl ethylamine(DIPEA) as a base. For each coupling, the ratio of amino acid, TBTU, HOBT, DIPEA is maintained 8:1:1:2 (in molar equivalents) with respect to the resin and bound amino acid and coupling is continued for 45 minutes. After each coupling, the residual uncoupled amino acid was capped by acetylation,

using acetic anhydride and lutidine in dry DMF, followed by repeated washing in dry DMF. The peptide was cleaved from the resin, and the side chain protecting group are removed by incubating in deprotecting reagent (Trifluoracetic acid: phenol: thioanisole: H2O: ethanedithiol= 82.5:5:5:5:2.5) for 3 hours at room temperature. The peptides were purified by reverse phase HPLC on microbondapac C-18(Waters associates) column using 0-30% acetonitrile in 0.1%TFA gradient over 30 minutes. The synthesized peptide was characterized by ESI mass Spectrometry in the positive mode.

7.1.1.3. Coupling of CSP nanoparticle with the peptide

CSP (10mg) is dispersed in 20ml of water/PBS buffer and to that EDC (10-20 equivalent with respect to peptide) and sulpho NHS (10-20 equivalents with respect to peptide) are added. In it, 200 ug peptide was added dropwise and the reaction is stirred for hours. Then the suspension is centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and the pellet was washed with 15 ml of water and centrifuged again at 10,000 rpm for ten minutes for the removal of extra peptides and other reactants. The washing process is repeated 3-4 times and each time the supernatant is collected. The supernatants and the pellets are dried in a lyophilizer.

7.2. Characterization of peptide tagged with CSP nanoparticle

7.2.1 Measurements of zeta potential

In the measurement of the zeta potential of the peptide, the free CSP particle is used as a control against which the zeta potential of the CSP-tagged peptide is measured. Both the free CSP and CSP-tagged peptides were dissolved in filtered HPLC water and submitted to the elemental analysis at the Ballygunge Science college.

7.2.2 Elemental Analysis

Dried CSP and dried CSP-tagged peptide in 1.5 ml Eppendorf tubes were submitted central instrument facility of Indian Association for the Cultivation of Science for the elemental analysis.

7.3. Results and discussions

Figure 7.1: Sequence of the linear peptide

The peptide sequence is shown in Figure 7.1. First, this peptide was synthesized using Rink amide PEGA resin in 0.1 mmole scale. For the first coupling, the substitution of the resin was fixed by using the procedures mentioned in chapter 2. After that, the successive amino acids were linked using six times molar excess of the amino acid with respect to the resin-bound one, using TBTU as the activating agent and DIPEA as a base. After the synthesis was completed, the peptide was cleaved from the resin by incubating with 94% TFA, 2.5% EDT, 1.5% Thioanisole,

1.5% water, and 0.5% TIS for 3 hours at 25 °C. The crude peptides were purified by HPLC on a reverse-phase Hypersil Gold C18 column using 80% Acetonitrile in 0.085% TFA and water in 0.1% TFA. An isocratic gradient of 32% acetonitrile in 0.085% TFA and 68% of water in 0.1% TFA was run for 65 minutes and the pure peptide was obtained at about 32 minutes. The HPLC chromatogram of the linear peptide is shown in figure 7.2.

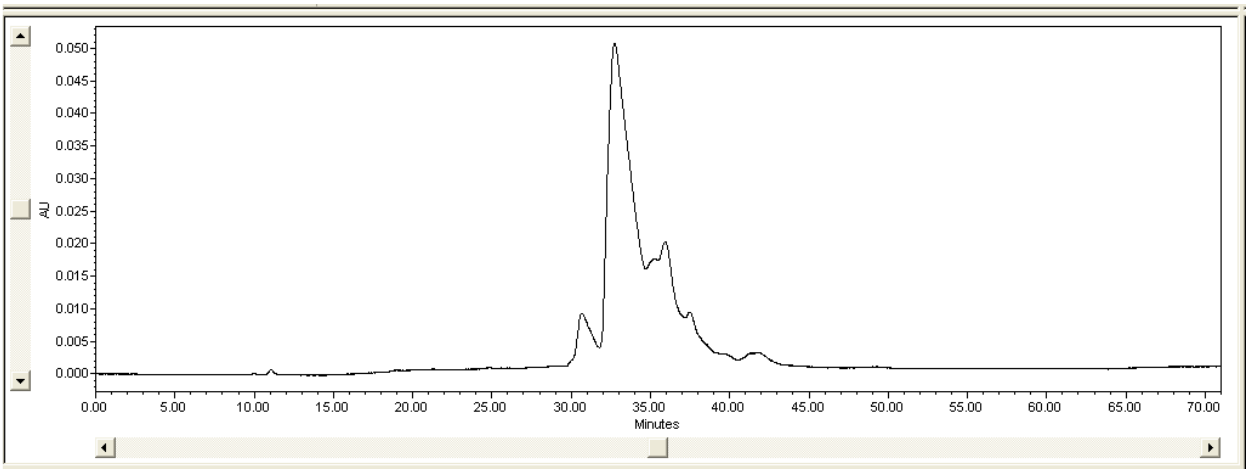


Figure: 7.2. HPLC chromatogram of the linear 7-mer peptide.

Table: 7.1. Mass of the linear peptide.

Name of the peptides	Expected mass	Obtained mass
Mass of the linear peptide	1009 daltons	1010 daltons

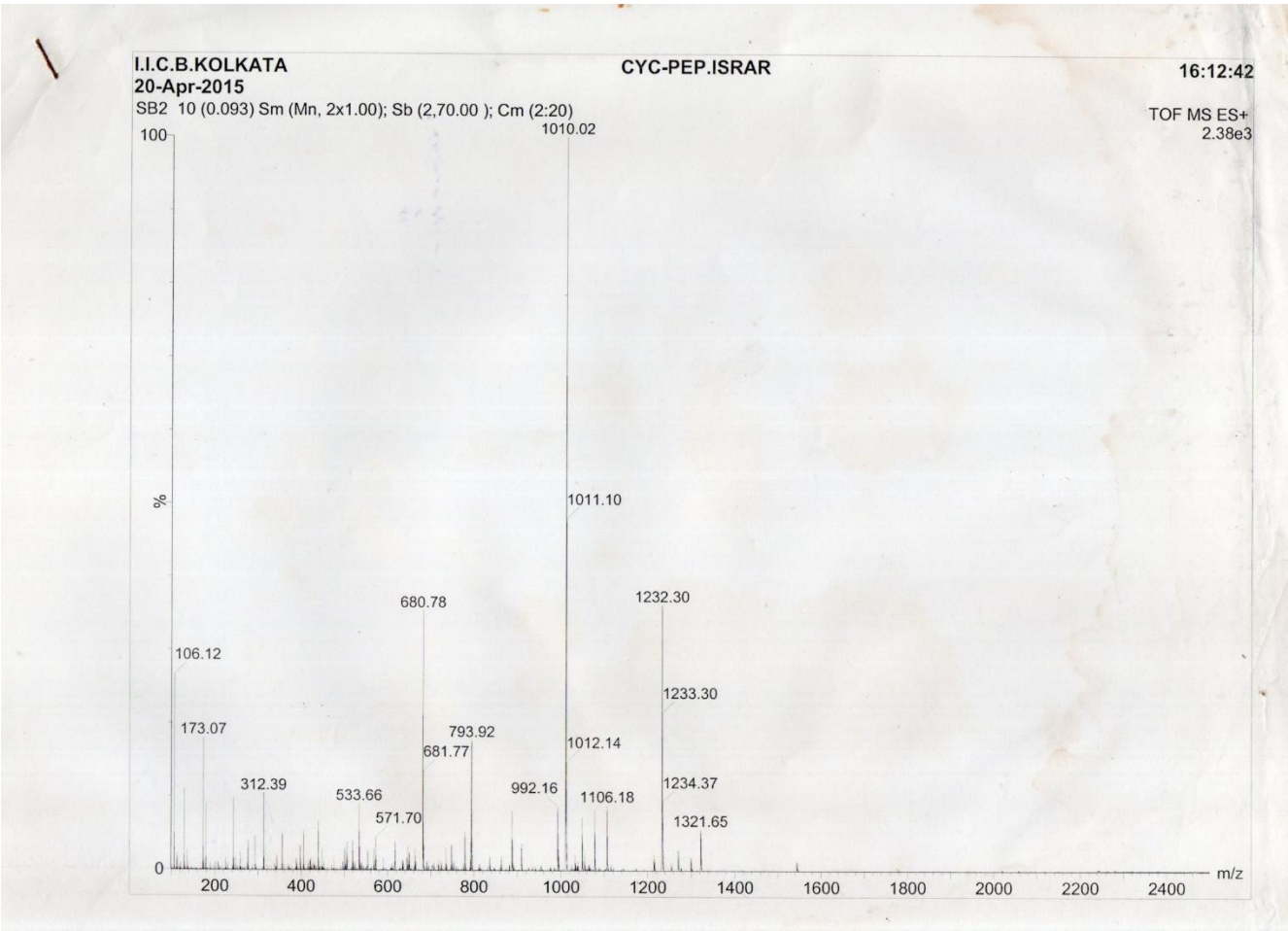


Figure 7.3. ESI mass spectrum of the peptide.

The mass of the peptide is shown in table 7.1 whereas the ESI mass spectrum is shown in figure 7.3 above.

The CSP nanoparticle functions as a cell-penetrating tag that directly enters not only the cell and even localizes into the nucleus without any other modification. For this purpose, the peptide was tagged with the CSP nanoparticle following a coupling process as described in the methods. The coupling of the nanoparticle with the peptide was ascertained by the measurement of the zeta potential of the peptide against the free CSP particle. Since the nanoparticle is a collection of negative charge on the surface containing the free carboxylate

anion, the coupling of the peptide with the nanoparticle will result in significant decrease in the negative charge of the peptide. This was confirmed by the zeta potential data in Figures 7.4 and 7.5 below. The charge i.e., obtained from the zeta potential data is shown in the table 7.2.

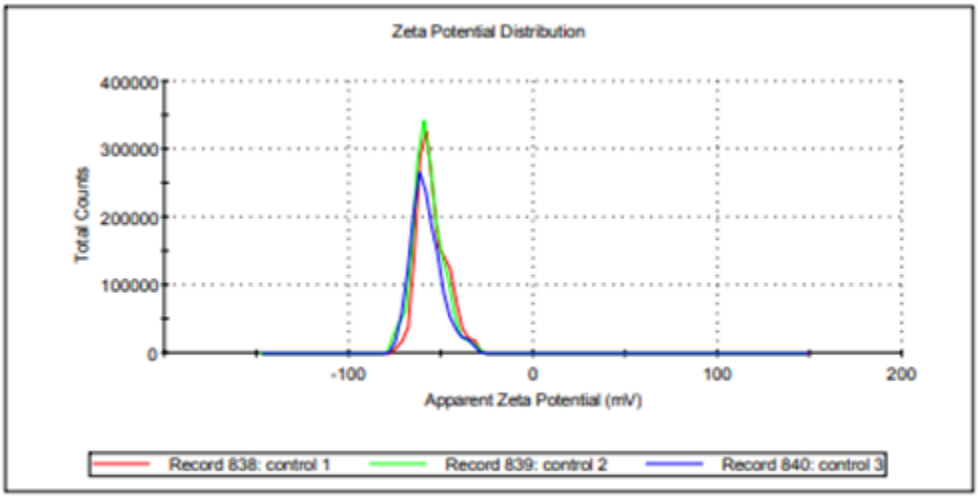


Figure 7.4. Zeta potential data of free CSP (gifted from Prof T.Kundu's lab) nanoparticle using as control

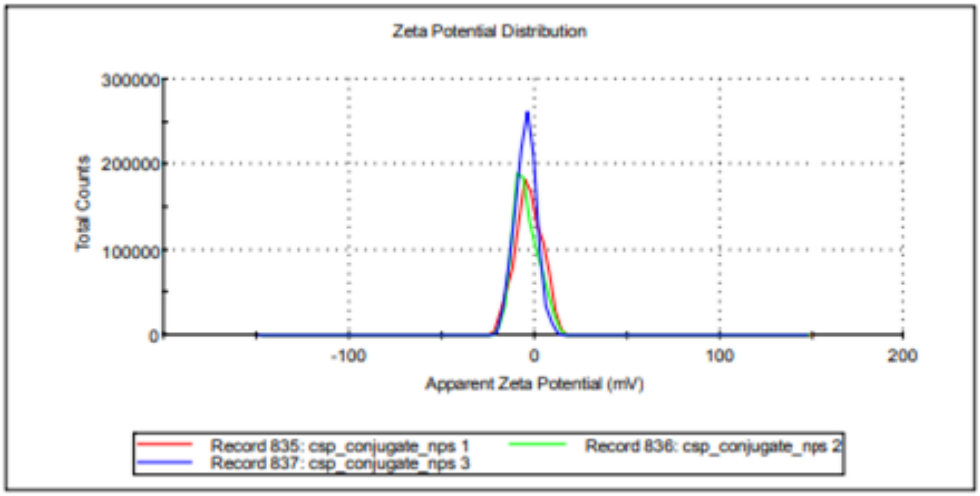


Figure 7.5. Zeta potential data of peptide coupled with CSP(gifted from Prof T.Kundu's lab) nanoparticle.

Name of the sample	Calculated Charge
Free CSP nanoparticle	-54.7
CSP nanoparticle coupled with the peptide	-3.18

The negative charge of the free CSP particle was measured as -54.7 on coupling with the peptide this charge was remarkably decreased to -3.18 which is due to the coupling of the peptide on the surface of the nanoparticle. Another method of characterization of the nanoparticle i.e., coupled with the peptide was ascertained by elemental analysis. The elemental analysis data of the free CSP particle and the peptide coupled with the nanoparticle also confirms the coupling of the peptide with the nanoparticle as shown in Table 7.3.

Sample	% Of Carbon	% Of Hydrogen	% Of Nitrogen
Free CSP Nanoparticle	27.85	3.06	0.00
CSP coupled with peptide	1.72	2.14	0.37

The free CSP nanoparticle shows the percentage of nitrogen almost zero as was expected since the CSP is based entirely glucose. The peptide coupled with the nanoparticle shows the percentage of nitrogen of 0.37 and this increase in the percentage of nitrogen comes from the peptide.

CHAPTER 8

A BRIEF SUMMARY: DISCUSSIONS & SIGNIFICNACE

In many tumors it has been observed that S100B protein is found to be elevated and serves as a biomarker of head injury. The tumor progression activity of S100B is due to its contribution towards interaction with wild type p53, thus downregulating and inhibiting its function as a tumor suppressor. It has been reported that pro survival pathway may also be regulated by S100B. Inhibition of S100B may simultaneously regulate several key growth regulatory pathways and exert broad antitumor effect. Overexpression of S100B is primarily noted in melanomas and gliomas in these classes of neoplasm it plays a crucial role in cancer development and progression. In the first part of my work an inhibitor of S100B is designed and

developed which can effectively and specifically blocked S100B. In this regard, a modified branched bivalent peptidomimetic of TRTK-12 sequence from the protein Cap Z (265-276 TRTKIDWNKILS) was synthesized which binds to the S100B at the same region with even tighter affinity than that of the original TRTK-12. To constrain the helical conformation of the peptide we have chosen substitution of helicogenic α- Aminoisobutyric acid (Aib) substitution as an alternative due to its simple synthesis methodology and possibly better scaling up potential for stabilizing helix. Using the structure of S100B-TRTK protein-peptide complex as a guide, we replace only those residues of the target peptide that are not interacting with S100B but are still part of the target sequence. A negative control of the peptide was synthesized in which two residues that directly interact with theS100B protein were substituted by Ala. The binding constant of the Ala substituted bidentate branched TRTK with the S100B decreases substantially.

IN another part of my work, the tissue distribution of the branched bivalent peptidomimetics in both wild type and tumor bearing mice has been investigated by chelating with radioactive ^{99}Tc by tricarbonyl method. Result demonstrated that in both normal and tumor bearing, considerable distribution of the peptide occurred in all the organ except for brain, heart, stomach, and large intestine. Most accumulation occurs in the liver and a large portion undergoes renal excretion. In tumor bearing mice, the doses reaching the implant tumor is small but noticeable. To improve the tumor doses of peptides, we have explored that the nanoparticle can be developed as a delivery platform. The peptidomimetic was coupled with a Carbon Sphere Nanoparticle (CSP nanoparticle is obtained as a gift from Prof. Tapas Kundu's lab). The characterization of the coupling of nanoparticle with the peptidomimetic was

done by measuring zeta potential. The CSP nanoparticle being negatively charged, and the result shows a remarkable decrease in the negative charge, ascertaining the coupling of nanoparticle with the peptide. An alternative way to ascertain the coupling of nanoparticle to the peptide is elemental analysis and the result shows the increase in percentage of nitrogen that comes from the peptidomimetics. Due to some unavoidable circumstances the in vitro study of the CSP nanoparticle coupled peptidomimetic is not yet been performed.

In the last part of my work two novel method of modifying the peptide for intracellular targeting has been described which will help in developing peptides as good therapeutic agent. In the treatment of β-hemoglobinopathies including β-thalassemia and sickle cell diseases one of the possible therapeutic strategies is to upregulate the fetal hemoglobin level which is functionally close to HbA. Developing peptide-based transcription factor mimic against critical transcription factor in the ϒ-globin gene regulatory network could be a novel approach as much off target toxicity can be avoided. In this regard we have designed a peptide-based transcription factor that links two helices of a zinc finger domain to produce a peptide that binds to the target DNA sequence with high affinity and a significant degree of specificity. To our knowledge this is the first example of a modified peptide that mimics DNA binding of a zinc finger domain. Giving the preponderance of zinc finger containing transcription factors in eukaryotes, these types of peptides, may be useful for investigating the biological effects of such transcription factors and may offer an avenue for peptide therapeutics, specifically targeting transcription factors. Synthesis of a berberine conjugate of a peptide is also reported. Berberine is highly effective in many chronic diseases including diabetes, hyperlipidemia, heart disease, cancer, and inflammatory diseases. A peptide having the core sequence KYKETDL was

derived from La protein shows significant RNA binding and antiviral effect against hepatitis C. The difficulty is that the linear peptides are degraded by cellular and serum proteases. To restrict this degradation, the peptide is cyclized and synthesized and finally conjugated with the chemically modified berberine. The study of bioactivity of this berberine conjugated peptide is planned in our future research.

Peptides has traditionally been assumed as poor drug candidate due to its unfavorable characteristics mainly regarding their pharmacokinetics behaviors that includes its rapid clearance from the circulation within minute to hour after administration. Proteolytic degradation in serum limits the drug half-life reducing its bioavailable concentration. In general, peptides are also unable to cross the cell membrane by themselves and therefore cannot inhibit intracellular targets. The membrane permeability of peptide drug depends on multiple factors including peptide length and amino acid composition. The low pH of the gastric juice and even the bacteria can cause peptide degradation. The presence of proteases in the blood stream reduce peptide to inactive metabolites. To overcome these challenges several strategies have been applied to peptides which will help to peptides to be developed as a good therapeutic.

The monumental achievements in the field of peptide therapeutic is the synthesis of insulin which helped in providing the approval of 80 peptide drugs worldwide. In the mid of 20^{th} century several life-saving bioactive peptides such as insulin and adrenocorticotrophic hormone were isolated from natural sources. During 1950 to 1990 more peptide hormones and their receptors with therapeutic potential were identified and characterized. Further synthetic peptides such as oxytocin, vasopressin, and recombinant human insulin began to be developed

in addition to natural peptides. Peptide therapeutic cover a wide range including urology, respiratory, pain, oncology metabolic, cardiovascular, and antimicrobial. Peptides has also been successfully employed in the prevention, diagnosis and treatment of tumors, cardiovascular and cerebrovascular diseases viz hepatitis, diabetes, and AIDS etc. As per report till February 2022 more than 170 peptides were in active clinical developments with many more in preclinical studies. The worked described here establishes new drug targets for developing peptidomimetics and new methodologies for the future development of this important area.

8.References:

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