SYNTHESIS, CHARACTERIZATION AND CYTOTOXICITY OF SHORT CATIONIC LIPOPEPTIDE LP24

Dissertation submitted in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

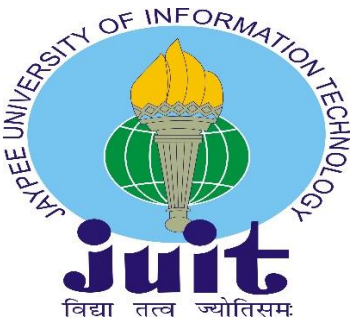
By

Amritansh Rai

207810

Under the Guidance of

Dr. Gopal Singh Bisht



JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY WAKNAGHAT

DEPARMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

HIMACHAL PRADESH-173234

DECLARATION

I hereby declare that the work reported in the M.Sc. dissertation entitle "**SYNTHESIS, CHARACTERIZATION AND CYTOTOXICITY OF SHORT CATIONIC LIPOPEPTIDE LP24"** submitted at Jaypee University of Information Technology, Wakhnaghat, Solan, Himachal Pradesh, India, is an authentic record of my work carried out under the supervision of Dr. Gopal Singh Bisht, Dept. of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Wakhnaghat, Solan, Himachal Pradesh-173234, India. I have not submitted this work elsewhere for any other degree or diploma.

Amritansh Rai, (207810)

Department of Biotechnology and Bioinformatics,
Jaypee University of Information Technology,
Wakhnaghat, Solan, Himachal Pradesh, India.

Date-

SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the M.Sc. dissertation entitle "**SYNTHESIS, CHARACTERIZATION AND CYTOTOXICITY OF SHORT CATIONIC LIPOPEPTIDE LP24**" submitted by Amritansh Rai (207810) at Jaypee University of Information Technology, Wakhnaghat, Solan, Himachal Pradesh, India, is bonafide record of his original work has not been submitted elsewhere for any other degree or diploma.

(Signature of Supervisor)

Dr. Gopal Singh Bisht

Associate professor

Dept. of Biotechnology and Bioinformatics

JUIT, Wakhnaghat,

HP- 173234, India.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the guidance and the help of several individuals who are one way or another contributed and extended their valuable assistance in the preparation and completion of this study. It is pleasure to convey my gratitude to them all in my humble acknowledgement.

First and foremost, it gives me immense pleasure and privilege to express gratitude to my supervisor associate professor **Dr. Gopal Singh Bisht**. I would like to acknowledge him for dedication, perseverance, patience and availability throughout this semester, the scientific expertise, as well as the constructive criticisms and suggestions made during the guidance of the work. Above all the most needed, he provided me with unflinching encouragement and support in various ways whilst allowing me the room to work in my own way, has inspired me to become and independent researcher and helped me realize the power of critical reasoning.

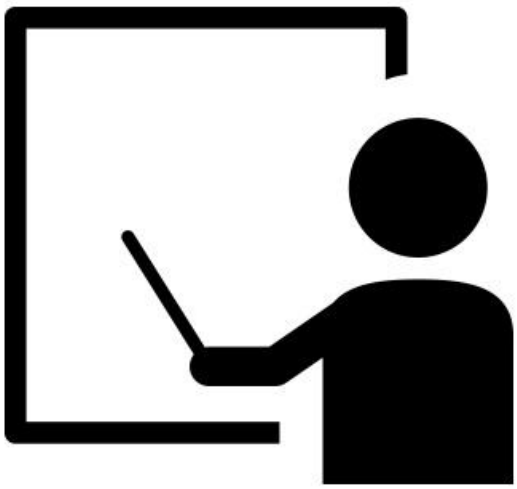
I am deeply grateful to **Ms. Lalita Sharma** for her humble guidance to me in this work. Without her knowledge and help in every step of work this research could not be possible. Special thanks to **Mr. Baleshwar Prasad** and **Mrs. Mamta Mishra** for providing me all the help with chemicals and reagents and their immediate availability to guide me in this work. I would like to thank to **Prof. Dr. Sudhir Kumar (HOD**), Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Wakhnaghat, Solan, Himachal Pradesh, who encouraged me motivated me for the work. I want to thanks for all the tip-offs and fair comments for this work.

List Of Abbreviations and Symbol:

3-ABA	3 Amino Benzoic Acid
ACP	Anti-cancer peptide
AA	Amino acid
Boc	tert-Butyloxycarbonyl
Conc.	Concentration
°C	Degree centigrade
C-	Carbon terminus
µg	Microgram
µL	Microliter
µmol	Micromole
DIC	Diisopropylcarbodiimide
DCM	Di-chloro methane
DMF	Dimethylformamide
Fmoc	Fluorenylmethyloxycarbonyl
HPLC	High-performance liquid chromatography
HOBt	Hydroxy benzotriazole
TFA	Trifluoro acetic acid
TLC	Thin layer Chromatography
TIPS	Tri isopropyl silane
N-	Nitrogen terminus
SSPS	Solid phase peptide synthesis
RBF	Round bottom flask

Contents

CHAPTER 1 INTRODUCTION



1. INTRODUCTION:

In the developing world, cancer is also increasing at an exponential rate. Alone in the year 2020 it is responsible for nearly 20 million deaths. Over the time, various forms of cancer have been brought to light. Currently, there are 100 types of cancer which affect the human race [1]. Various reasons are responsible for cancer but some major cancer-causing agents are tobacco responsible for 20% overall cancer death, other agents include diet, alcohol, environmental pollution, exposure to radiation etc. [2]. Basically, for development of cancer various genetically changes occurred for cancer. Nearly 5-10 percent of cancer is caused by inherited genetic changes [3]. Cancer is already a concerning problem due to its growth and death rate every year but there are two main problem which are firstly, treatment of cancer and secondly, diagnosis of cancer at beginning stage to prevent its further growth. Both of these issues are currently being studied and dedicated efforts are being made to develop newer treatment regime for cancer.

Conventional methods are not adequate nowadays to treat cancer completely and in large number of cases reoccurrence has observed. To overcome these problems currently different studies are going for treatment of cancer such as small molecule therapy, hormonal therapy, immunotherapy etc. in such studies one most effective treatment is Peptides role in cancer treatment. Peptides are extremely versatile tools with enormous promise for developing cancer diagnostics and therapeutics. Peptides are amino acid chains that are short and linear (AA). They are typically 50 AA long and are frequently stabilized by disulfide linkages. They are intentionally designed with great specificity to bind and control a particular target [4]. Therapeutic peptides offer various benefits over proteins or antibodies, including their tiny size, ease of production, and ability to enter cell membranes. They also exhibit high activity, specificity, and affinity, as well as little drug-drug interaction and a wide range of biological and chemical variety. Another advantage of employing peptides as a medication is that they do not store in certain organs (such as the kidney or liver), which can assist to reduce hazardous side effects[5]. Peptides can be used for cancer treatment in a variety of ways, including mimicking natural proteins to either boost or inhibit signaling, targeting medicines particularly to cancer cells, and employing them as a tool for trans barrier delivery [6].

In the recent year the direct use of peptide for treatment of cancer is gaining momentum, and there are several promising advantages for using peptides as cancer treatment such as size, availability, modification, specificity [7]. Peptides using for cancer treatment are termed as Anti-cancer peptides. Anticancer peptide is a term which separates the peptides derived from antimicrobial peptides but has anti-proliferative activity on cancerous cell [8]. Indeed, cationic peptides derived from various species were previously tested for antibacterial activity and evaluated as such before being reported as anti-cancer drugs in 1985 [9]. The main property which distinguishes between AMP and ACP is their target of action. Anti-cancerous peptides act on cancerous cell either via apoptosis or through necrosis [10]. Most ACPs target the highly negatively charged cancer cell membrane initially by electrostatic contacts, then destabilize and break the cell membrane via hydrophobic interactions, resulting in cancer cell necrosis [11]. Another mode of action is the triggering of apoptosis in cancer cells via mitochondrial membrane rupture by ACPs. When ACPs interact with eukaryotic cells, they can cause mitochondrial permeability and enlargement by damaging mitochondria, leading in the release of cytochrome c (Cyt c), which causes cancer cells to die by apoptosis [12].

Peptide Name	Cancer Cell	Mode of action	References
Temporin-1CEa	Bcap-37, MCF-7,	Necrosis	[13]
Cecropin A and B	RT4, 647V, J82, 486P	Necrosis	[14]
Pardaxin	Hela, HT1080, MBT-2	Apoptosis	[15]
Mellitin	L1210, U937	Necrosis	[16]
BMAP-28	U937, K562	Apoptosis	[17]

Table 1.1: Anticancer peptide and their mode of action

Apart from apoptosis and necrosis various other model were proposed for working action of peptides on membrane permeation. Some of them are most studied and accepted mode such as Carpet Model, Barrel Stave formation and Toroidal pore method. In the Barrel Stave pore formation peptide interact with membrane in perpendicular direction such that hydrophobic part of peptide penetrates cell membrane and forming pore in cancerous cell. Due to pore formation the cancer cell are destroyed by necrosis [18]. Such pore forming models are generally describes the working of the membranolytic peptides. Alamethicin and paradaxin are peptides which shows such mode of pore formation [19]. Another method of pore formation by peptide is carpet model in which peptide accumulates near membrane and when this accumulation reaches a critical concentration then pore formation occurs. Basically, according to this concept, lytic peptides initially attach to the target membrane's surface and blanket it in a carpet-like fashion. Membrane penetration occurs only when the local concentration of membrane-bound peptide is high. In contrast to the 'barrel-stave' concept, peptides are not introduced into the hydrophobic core of the membrane in the 'carpet' model. In the carpet model specific structure adoption is not required when binding with the membrane as in Barrel stave model [20]. Peptide with such mechanism are cecropin dermaseptin aurein and citropin from frog skin secretions [21]. In the toroidal pore model, the hydrophilic portion of the peptide interacts with the lipid head, generating a local membrane curvature that lines the pore lumen partially with peptide and partly with lipid heads [22]. This model, sometimes known as a "two stage" model, where peptides are aligned parallel to the plane of the bilayer at low concentrations (inactive state) when the peptide molecules reach a certain concentration, they are reoriented perpendicularly, breaching the bilayer's hydrophobic area (active state) [23]. Magainin and melittin from bee venom has been shown to form a toroidal pore [21].

1.1 Therapeutics properties of peptides:

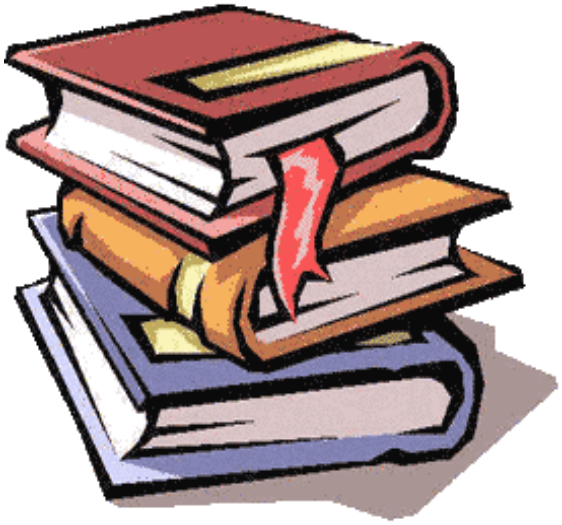
Various properties of peptides make them effective in treatment of cancer such as broad and specific range of target, low toxicity, safety with good efficacy, also their low accumulation in tissue and organ, having standard protocol for synthesis. One of another important properties of these therapeutic peptides that they can inhibit the interaction between oncogenic proteins because structure and design of different oncogenic protein and their interaction are already known [24]. For example, if the sequence of the binding site of two proteins is known, a peptide can prevent this interaction [25]. In the studies it was found that Magainin 2 shows anti-proliferative properties on bladder cancer cell but no effect on human

fibroblast shows the specificity of peptides [26]. Buforin IIb was found to be cytotoxic to human cervical carcinoma (HeLa) and leukaemia (Jurkat cells) cells in vitro, as well as inhibiting the development of human lung cancer xenografts in mice by inducing apoptosis in cancerous cell membrane [27]. Peptides in cancer treatment can also be use as LHRH (luteinizing hormone-releasing hormone) which is a antagonist for prostate cancer therapy [28]. Tumour-associated antigen are expressed by tumour cell which is recognized by host immunes system. Many TAAs have previously been discovered and characterized [29]. These TAAs can be injected into cancer patients in order to trigger a systemic immune response, which may result in the death of cancer cells developing in different tissue or organ. This treatment is classified as active immunotherapy or vaccination since the host's immune system is either triggered from scratch or restimulated in order to establish an effective, tumor-specific immune response that may result in tumour regression. Protein/peptide produced by cancerous cell has mutated structure due to mutation and this can act as antigen for tumour. This peptide produced by cancerous cell can be used as therapeutics in treatment of cancer [30]. Various other properties of therapeutic peptides are discussed which makes peptides better option of choice for treatment in cancer.

Advantages of Peptide	Disadvantage of peptide
Specific and broad spectrum of target	Biological instability
Low toxic effect	Structure sensitivity by proteases
Biological and chemical diversity	Short half life
Efficacy and high potency	Poor/ slow absorbance
Less accumulation in organ and tissue	Poor pharmacokinetics
Standard protocol for synthesis	Limited oral availability
Selectivity, safety and tolerability	

Table 1.2: Advantages and disadvantages of therapeutics peptides

CHAPTER 2 REVIEW OF LITERATURE



2. REVIEW OF LITRATURE:

Cancer is the uncontrol growth of the cells and which grow from other body part. According to the stats the new cases in the year 2020 was 18.6 million globally and nearly 9.65 million deaths globally. According to this data the current medication and the treatment for the cancer is still not adequate because still there is large number of the patients for cancer and the death ratio is increasing year by year [31].

In the current scenario for the treatment of the cancer number of the treatment goes simultaneously which includes the surgery, radiotherapy, chemotherapy and immunotherapy, bone marrow transplant etc. In general, surgical treatment can remove the visible solid tumours; this could be a useful therapeutic technique for early or even intermediate tumours. However, because this type of treatment frequently results in visible injuries, bleeding, infection, decreased immunity, and other hazards, it is not the best option for the majority of patients with advanced tumours [32]. Also, in the surgical removal of the cancer not only the cancerous cell but also the normal cells are removed. In radiation therapy is frequently utilized in cancer patients who have not responded to surgical treatment. In this therapy uses high power energy beams on the cancerous cell such as the X-ray or protons to kill the cancer cell. However, radiotherapy is associated with a number of problems such as anaemia, hair loss drastic loss of weight, trouble with the memory and it is also a costly therapeutic approach with a lengthy treatment period [33]. Chemotherapy is a series of therapy in which medicine and chemicals are given to patient to kill cancer cells. But the problem arises after the long-term use of medication when the resistance against the drug develops and chances of the recurrence of the cancer is also likelihood; also, medicines kill tumour cells and normal cells both , due to which sometimes the results are more adverse than the treatment.

Caner survives in the body because it remains unchecked by the immune system so in thew Immunotherapy act in such a way that the patient's self-immune system to initiates an anti-tumour effect. This treatment is effective as compared with the other treatment as it helps into activating the immune system which destroy the cancer cells, but still the affect and the treatment varies from the person to the person and sometimes it can also lead to the autoimmune disorder [34]. In the recent years the small molecules are also used for the cancer treatment such as the C188-9, a STAT3 inhibitor, in head and neck cancer, and GNS561, a lysosomotropic molecule, in bile duct cancer [35].

In summary, clinical treatment approaches each have their unique set of benefits, drawbacks, and applicability ranges. A single treatment strategy can be difficult to generate adequate therapeutic benefits, and combined therapy can, to some extent, provide a superior overall cure. But till date no such therapy or medication available to claim for the better and complete treatment for the cancer. As a result, there is an urgent need for studies and development of therapeutic approaches for anti-tumor medications which can effectively act on the cancer and also changes the conventional method for cancer treatment.

In the last decade lots of research and studies has been done on peptides and their role in various aspects and over the years peptide has emerged as better therapeutic agent in the treatment of cancer. A large number of peptides are currently in the clinical trial for the treatment of 36 cancer types and some unclassified type (such as solid tumour, neoplasm). These peptides are known as the anticancer peptides, which are derived from the antimicrobial peptide but they have cancer related toxicity. And most important feature of these anticancer peptides is that they can distinguish between the normal and the cancerous cell which makes them more selective and toxic for the cancer cells.

We will further discuss about all these properties and about anticancer peptides:

2.1 Anticancer Peptides:

With the recent advancement in the molecular biology, it has been found that the large number of the short peptides are present and produced by the organism which can kill the bacteria, fungi and even the tumor cells, also help in regulating the immune system. Anticancer peptides are the small peptides which contains the specific amino acid sequences, low molecular weight cationic peptides which are selectively toxic to the cancer cells [36]. Due to their high and specific selectivity, high penetration and modification to increase their effectiveness also resistance in ACP is very low as compared to conventional drug therapy low production cost makes ACP superior choice for the therapeutics compared with other treatment for cancer [37]. From the structural point of view ACP can be either the α-helical or β-sheet conformation but some other extended structures have also discovered [38]. Also based on their target they can be classified in which the first categories include the peptides that work against the cancerous cell and not the healthy mammalians cells such peptides are cecropins and magainin's, whereas the second category consist of the peptides that work against cancerous cell, healthy cell and microbial cells. Human neutrophil defensins HNP-1 to 3 is example of such peptide[39]. Anticancer peptide should show antiproliferative activity

on a range of cancer cell, but not on healthy cell membrane. and are simple to make the modifications. According to research in National Institutes of Health Clinical Trials database 1002 peptide-based clinical trials that targeted different types of cancer are being studied and researched [40].

2.2 Working Mechanism of ACP:

The differentiating factor for ACP in cancer and normal cell membrane is presence of negatively charged molecule on cancer cell and healthy cell membrane are neutral in nature. This difference in cell membrane leads to membrane destabilization, cytotoxicity and cancer cell lysis when peptide bind to cancerous cell [40]. This also affect the interaction between cell membrane and peptide i.e. for cancerous cell this interaction is electrostatic and for healthy cell have hydrophobic interaction with peptide [41]. ACP recognize the difference between the cell membrane and then destroy the cells either via the apoptosis mechanism or necrosis and in both mechanism peptide ether destroy cell membrane or damage mitochondria [38]. Cell membrane also play an important role in the binding or inhibiting peptide penetration and binding, also the peptide conformation i.e. alpha helical or beta sheet and there interaction with membrane either perpendicular or parallel affect the peptide therapeutics [42]. In healthy cell membranes have the membrane configuration as zwitterion phosphatidylcholine and sphingomyelin in an outer leaflet and anionic phosphatidylserine and the phosphatidylethanolamines in the inner leaflet with the asymmetric distribution [43]. This asymmetric is maintained by the flippases and the floppases. And due to this asymmetric distribution, the healthy cells have the net neutral charge on the cell surface but the cancer lost this property and due to which it has the negative charge of phosphatidylserine exposed on surface of membrane, as well as the locating of phosphatidylethanonlamine on the outer membrane of cell [44]. Due to all this changes in the cell membrane promotes the drug penetration. Also, the pH of the cancer cell changes from the 7.4 to 6.5 and because of this pH change surrounding environment becomes acidic promoting cancer metastasis [45]. Normal cells protect themselves from the lytic activity is due to the presence of the cholesterol in the cell which modify the cell membrane fluidity. Also, the cancer cells have the large number of the microvilli as compared to the normal cells due to which their cell surface increases [46]. Peptides working can be either membranolytic or non-membranolytic depending upon interaction with membrane and also nature or configuration of peptides[21]. Alpha helical peptides are commonly membranolytic peptides due to which they interact with cell membrane and causing pore formation in membrane by different methods such as Stave

barrel or toroidal pore, which leads the change in conformation of cell membrane causing necrosis of cancerous cell [47]. In the non-membranolytic method peptide penetrate cell membrane and cause damage to intracellular targets such as DNA, RNA or mitochondria where peptides damage the mitochondria inducing mitochondria swelling and release of cytochrome C which leads to apoptosis of cell(programmed cell death) [12]. Currently different research and studies are conducted to understand more clearly the working mechanism of peptides and there interaction so that more specific and high anti-proliferative activity of peptides can be achieved in therapeutics.

2.3 Role of Amino Acid in the Anticancer Peptides:

Amino acid composition plays the important role in the working of the anticancer peptides. The predominate amino acids that are present in the ACP are glycine, lysine and leucine [48]. The lysine and arginine are hydrophobic positively charged and due to which they act as the cationic peptides, and since the cancer cells are negatively charged, they can easily penetrate the cell membrane and serving a role in cancer cell toxicity [49]. Histidine, Glutamic and aspartic acid also play the role in the initiates the anticancer activity on the tumour cell either by the necrosis or apoptosis [50]. Some amino acids such as the serine and the glycine free diet can also induce the anti-proliferative activity and slow the tumour growth [51]. Methionine increased levels can be consumed by the cancer cells otherwise they do not play any direct role in the supressing the activity of tumour cell. Methionine deficit diet can help in arresting cancer cell growth as it causes a metabolic defect in cancerous cells [52]. Phenylalanine is strong hydrophobic amino acid mainly present in primary tumour [53] and peptides containing Phenylalanine increases the affinity for targeting and binding with cancerous cell [53]. Tryptophan and tyrosine also have the role in the ACP both are the weak hydrophobic amino acid but if the tryptophan and histidine are present together then they decrease the cytotoxicity of the ACP, and peptide with amino acid residues such as proline tyrosine, phenylalanine may be able to enhance the cytotoxicity of the peptides. The position of the amino acid also has the impact on the penetrating the cell membrane and interacting with the cell. In studies found that arrangement of two tryptophan helps into regulating cellular entry and transporting the peptide from cytoplasm to nucleus. A four peptide residue sequence of Glu-Thr-Trp-Trp shows that Trp position help into enter cancer cell where it binds to the nuclear DNA and shows it ant proliferative activity [54]. The peptides amino acid content can directly affect on numerous cancer cell types. Highly cationic peptides, for example, can boost cancer cell specificity, but an increase in hydrophobic peptides can

reduce specificity [55]. Apart for the role of amino acid various other factors are also responsible in the designing of the anticancer peptides such as the amino acid sequence, hydrophobicity of peptide, secondary structure and orientation, length of the peptide, isoelectric point, molecular weight, charge on peptide [56].

Amino acid	Properties	Effect on cancer cell	Reference
Arginine and lysine	Basic and cationic Amino acid	Shows anti-proliferative activity by penetrating and disrupting cell membrane.	[49]
Cysteine	Non charged amino acid and polar nature	Helps into maintaining the structure and stabilization of peptide.	[57]
Glycine	Most basic and common AA in ACP	Helps into membrane interaction and permeability.	[58]
Phenylalanine	Hydrophobic and aromatic	Help into increasing the affinity for cancerous cell and may also increase cytotoxicity.	[59]
Methionine	Non charged and non-polar AA	Shows anti-proliferative activity on cancerous cell	[52]
Histidine	Positively Charged, polar and hydrophilic AA.	In acidic condition increases anti-proliferative active of peptide	[50]

Table 2.1: Role of different amino acid in ACP with their properties.

2.4 Classification of anticancer peptides:

Based on the various organism the different type of the anticancer peptides is found in the nature and some are designed according to their working [60]. Due to large number of peptides found they are categorized accordingly

2.4.1 Based on the Structure:

α-helical ACPs: It is the most common and abundant type of the ACP that is found and they are small in the length and have basic structure. These types of the ACP are commonly found in the Amphibians in the epidermis part [61]. Magainin 2 found in African frog was first α-helical peptide which has anti-cancer activity [26]. Aurein is α-helical peptide also found in frog such as golden bell frogs [62]. α-helical ACPs are most studied in the anticancer peptides and they have acceptable anti-tumour effect. The α-helical ACPs are basically rich in the amino acids such as the lysine and the arginine and due to which they have the net positive charge at the neutral pH. The number of the amino acid are usually from the 5-20 amino acid in α-helical ACPs. Different organism shows the different half maximal inhibitory concentration on the cancer. Various modification was made in the α-helical ACPs to increase their stability and their anti-tumour effect [7]. The exact mode of the action for the α-helical ACPs are not found but the interaction between the cell membrane and the α-helical ACPs cause the cell lysis and the cell death.

β-pleated sheet ACPs: These ACP are usually found in the both plant and the animal. β-pleated sheet ACPs are formed parallel or anti-parallel stabilized by the hydrogen bond which lead to the amphipathic structure in which both hydrophilic residues are at one side while at the other side hydrophobic residues are present in chain. In the β-sheet peptide they have two or more sulphide bonds which help into providing good stability to structure [63].Some examples for the β-pleated sheet ACPs are such as the Bovine lactoferrin (LfcinB) [64], Human neutrophil peptide (HNP-1) which has a strong inhibition effect on the human prostate cancer [65]. Anti-proliferative activity of alpha helical peptide are more than β-pleated peptides but β-pleated are less toxic to normal cell and due to which they are more preferred for therapeutics.

Random coil ACPs: Random coil ACPs are formed when the monomers subunits are joined randomly but they are bonded to the adjacent units. They are rich in the proline and the glycine amino acids but they do not have the secondary structures [66]. Alloferons, a glycine amino acid rich random coil ACP obtained from insects, can induce NK cell activation and interferon generation in animal and human models, enhancing antiviral and anti-tumour properties in mice and people [67]. Random coil ACPs had a weaker lethal effect on normal cells than other types of ACPs, and their inhibition of tumour cells is much lower than α-helical ACP and the β-pleated sheet ACPs. So, improving their anti-proliferative activity and increasing cytotoxicity for cancerous cell is still under studied and researched.

Cyclic ACPs: Cyclic ACPs are circular and closed loop peptides with a cyclization from head to tail backbone or cystine knots form because of the disulphide bonds they are more stable compared with linear structures peptides [68]. Diffusa Cytide1,2 and 3 are novel cyclic peptides discovered in the roots and leaves of the white snake plant that have a substantial inhibitory impact on prostate cancer cells [69]

The cyclic ACP has the majority for the study as it has the strong inhibition effect on cancerous cells [70]. H-10, a new cyclic pentapeptide, inhibits mouse malignant melanoma B16 cells in a concentration-dependent manner, with an IC50 value of 39.68 M and no toxic effect on human peripheral lymphocytes or rat smooth muscle cells [71]. Cyclic peptides has better anti-tumour activity with comparative low toxicity than other peptides and can have better therapeutics in future with modification.

2.5 Based on their mode of action:

Molecular targeted peptides: ACP are designed or found with specificity to choose and destroy cancer cells by penetrating the cell or by binding to cell membrane and then destroy the cancer and the cancerous cell that are at the important stage of the proliferation [72]. They are categorised into the two groups as: 1) Peptides that act only against the cancerous cell and not the healthy cells and 2) peptide that work against both the healthy and the cancerous cells [73]. Large number of the peptides are there which act only on the cancerous cells such as the defensins, chrysophsin-1, lactoferricin B, magainin and cecropins [56]. Based on the method of peptides mode of action on cancerous cell they are divided into three different categories such as: Pore-forming peptides, which attach to negative charges molecules on the carcinoma cell membrane, causing apoptosis or necrosis of cancerous cell; cell-penetrating peptides,

which moves across the plasma membrane, transporting small molecules proteins from cytoplasm to nucleus, a process known as internalisation and then damaging intracellular targes; and tumour-targeting peptides, which bind to receptors on the cancer cell surface and cause anti-proliferative activity [10]

Peptides as Guiding missile: These binding peptides are also known as guiding peptides which does not have any direct effect on the cancerous cells but they carry the anti-tumour medicine bind to them to the specific target sites. The main property for guiding missile peptide is that they should be hold the targeted medicine loosely, should get dissolve and release the drug inside tumour cell membrane [74]. These peptides should also have the specificity, affinity and dose effectiveness toward drug [75]. The main problem with drug transport is that concentration of the drug decreases as transported towards the targeted area. But with help of the binding adjuvant and nano particles the concentration of drug can be retained during the transport to the targeted area and also help into delayed release of bind drug at target site [76]. Without the involvement of specialised receptors, amino acid sequences of cell-penetrating peptides, consist of 5-30 amino acid residues, notably positive residues, that can penetrate through tissue and cell membranes. For protecting the drug from degradation by enzymes peptides are attached with drug either by covalent bond which is primarily disulphide and thioester bonding or non-covalent bonds such as hydrophobic or electrostatic interactions [18]. Binding peptides can be divided into three groups based on their physical and chemical properties: Peptides which are cationic, amphipathic, or hydrophobic [77]. The physiochemical properties of peptides such as concentration and size of peptide play role during binding peptide enter the target cell either by formation of pore and destabilizing membrane or by endocytosis [78].

2.6 Anti-proliferative activity of ACP:

2.6.1 Destabilizing the cell membrane:

The cell membrane of the cancer cells is destroyed by the ACP due to which the cytoplasmic content of the cell as the normal osmotic pressure cannot be maintained by the cancerous cell [79]. Most of the ACPs has this working mechanism of destroying the cell membrane and due to which they have the advantage over the chemotherapy as they can destroy the cancer cells such as growing one, or the small tumour even the multi drug resistant tumour can be destroyed by the ACP [80]. HPRP-A1-TAT, a hybrid peptide, which cause leakage of

cytoplasm due to destruction of cell membrane with the IC_{50} value less than 10 μM for gastric cancer, cervical and liver cancer [81].

2.6.2 Inhibition of tumour angiogenesis:

Development and metastasis of cancer is promoted due to the high level of Vascular Endothelial growth factor which is also responsible for generating new blood vessel for cancer cells. Due to this new blood vessel development the tumour cell which don't have the blood vessel supply also gets connected to new blood vessel [82]. In apolipoprotein (A) KV domain a peptide with 11 amino acid KV11is present which reduced the angiogenesis inhibiting migration of human umbilical vein epithelial cells and also formation of microtubules. ACPs work by inhibiting neovascularization rather than killing tumour cells, therefore they have few negative effects on normal cells. As a result, ACPs of this type have a promising therapeutic future.

2.7 Modification of the Anti-Cancer Peptides:

Anti-Cancer peptide have the potential for the cancer treatment ability but they also have the side effects, decreased actions against the tumour cells and the stability which affect their use in the clinical and research applications. Several researchers have focused their efforts in recent years on the reconstruction and modification of ACPs in the hopes of preserving their benefits while lowering their associated negative effects, hence increasing their therapeutic characteristics. Basic chain rearrangement and side chain modification are the two main types of ACP reconstruction. The substitution of both non-natural and natural amino acid residues is referred to as main chain reconstruction, where different modification such as cholesterol , phosphorylation of peptide, polyethylene glycol (PEG) modification, glycosylation, and palmitoylation are some modifications.

2.7.1 Main chain Transformation:

The working and structure of small peptides are heavily influenced by the kind and amino acids residue sequence. As result, most common and successful technique to modify ACPs is to change the kind of main chain amino acids. ACP activity and selectivity are affected by changes in total charge of peptide chain, hydrophobicity, and helix of peptide by different amino acid substitution (45). Tumour cells are negatively charged than normal cells, but peptides are cationic charged and bind with cell membrane better. Furthermore, the

interaction between the surface of ACPs which is hydrophobic and phospholipids in extracellular domain of tumours can be rather powerful; a careful balancing of these various actions could significantly improve ACPs' anti-cancer effectiveness (46). To explore the influence of total charge on ACP activity, the peptide K7S was modified on the hydrophilic surface by replacing the two amino acid residues of lysine and serine to produce a peptide chain with total charge of +4 to +10 while no change in hydrophobicity of peptide. Due to this modification studies shows that IC_{50} value for cervical cancer decreased from 13.2 µM to 1.4 µM and due to which toxicity for normal cell also reduced. Changing the charge, hydrophobicity, and helical structure of ACPs is currently a routine practise to improve their anti-tumour action. However, the actual structure–function link is still unknown, which explains why present research on ACP modification is ineffective and might be the subject of future study. Non-natural amino acids frequently have physio-chemical qualities that native amino acids do not. As a result, these characteristics can be exploited to create short peptides with unique features that interact better with cell membranes (48). Non-natural amino acids have three major benefits over natural amino acids: 1. They have a wide range of physiochemical characteristics 2. Non-natural amino acids provide increased metabolic stability and 3. Enable more control of the structural flexibility of peptides, improving the possibility for organism selectivity and potency (49). D-amino acid is another non-natural amino acid utilised in ACP modification. The use of d-amino acids to substitute l-amino acids resulted in alterations in the structure of the polypeptide, which lowered the haemolytic rate of normal cells and increased stability and inhibitory actions greatly (50).

2.7.2 Side chain modification of the Anti-Cancer peptides:

In the animal cell membrane cholesterol is important component as it protects the healthy cell from lytic activity. Inducing cholesterol into ACPs may cause peptides to self-assemble, making it easier for ACPs to enter cancer cells. So thus, modifying the Cholesterol in the ACP it may increase anti-proliferative activity and also help into reducing toxicity of anti-cancer peptides (51). Phosphorylation is a ubiquitous change of a protein or peptide that happens at multiple distinct phosphorylation sites in amino acids such as threonine, tyrosine, and serine and some other amino acids after it is created. Some other modifications are also done in the side chain of the peptides to increase their anti-tumour effects such as the Polyethylene glycol modification, Glycosylation modification.

2.8 Future Aspects of ACPs:

In the current scenario, cancer is a major health concern worldwide and two main reasons are increasing number of cases every year and inadequate treatment available for cancer. Tumour cells can persist by evading the immune system of the host and producing proteins to withstand external therapy [83]. Traditional cancer therapies include chemotherapy, radiation, and surgical removal but each therapy have associated severe side effects and can create drug resistance, limiting its effectiveness. Due to these current medication problems, therapeutics peptides are gaining more attention and studies for their use in cancer treatment [84]. Basically peptides are chains of amino acid subunits connected by peptide bonds, can particularly adhere to tumour cells while being non-toxic to normal cells, which makes them essential in anticancer therapeutics [85]. Tumour targeting peptides depends on molecular structure for binding [85]. Various membrane protein is present on cancer cell membrane, such as endothelial cell growth factor receptors (EGFR) and cell surface proteoglycans, which allow chemicals to attach to these proteins precisely. Peptides produced from any source either it is natural or synthetic it can bind to these membrane proteins preferentially because they have similar structures due to presence of arginine and lysine. The amino acids binds with negatively charged cancer cell membrane by hydrogen bonds which also indicated that amino acid are responsible for binding of peptide with cell membrane [86]. Apart from these factors the confirmation of peptide also has importance in peptide therapeutics actions, as confirmation of peptide help in binding and initiating anti-proliferative activity.

Basically, various factor combined together plays an important role in peptide activity on cancerous cell. From the last decade when anticancer peptides and its role is discovered, continuous study and research is going for better understanding its role and its anti-proliferative property. Still there is large number of drawback in using peptide as therapeutic agents such as low bioavailability, biological instability, protease sensitivity, poor pharmacokinetics, short half-lives, minimum inhibitory concentration (MIC) etc. but apart from these disadvantages there are some major advantages in using peptides as therapeutics agents in cancer treatment which can completely change the cancer treatment scenario and due to which currently more than 1002 anticancer peptides are currently in different stages of clinical trials. Such as MUC1 peptide in clinical trail 1 has antiproliferative activity against breast cancer [87]. Numerous other peptides are also under ongoing research and in year 2012, 6 peptides were approved for marketing which encouraged more toward therapeutics peptides [88].

The advancement of modern medicine, research, and technology has resulted in the successful repair and alteration of ACPs. Nonetheless, each approach has its own set of restrictions. As a result, the ACP evaluation strategy should be sufficiently broad to achieve optimum efficiency. Constant investigation and finding answers to the various unfavourable consequences are required to develop a new ACP screening method. More study is needed to properly guide future modification and application development of certain ACPs. It is proposed that ACPs may encourage cancer treatment or vaccine development in the future to reduce new cases and fatality rates. Therapeutic's peptide in future can be breakthrough in cancer treatment.

CHAPTER 3 MATERIAL AND METHODS

3. MATERIAL AND METHODS:

3.1 Chemicals and reagents used:

Rink Amide MBHA resin and Fmoc-Orn(Boc)-OH protected amino acid purchased from Novabiochem, Mumbai. 3-ABA-OH and Fmoc-Cl were purchased from spectrochem, Mumbai. Lipopeptide used for synthesis is stearic acid purchased from Fluka, India. Other reagents used for synthesis such as 1-Hydroxybenzotriazole (HOBT) Piperidine N,N′-Diisopropylcarbodiimide (DIC) Dimethylformamide (DMF) from spectrochem, India. Dichloromethane (DCM), Diethyl ether and acetonitrile HPLC grade were procured from Merck, India. Trifluoracetic Acid (TFA) purchased from Loba chemie, Mumbai, India. For purification, all the solvents used were of HPLC grade and purchased from Merck, Mumbai, India. Other reagents and solvents used during synthesis were Triisoproplysilane (TIPS) ,Ninhydrin, Distilled water and MiliQ water.

3.2 Solid Phase Peptide Synthesis:

For the synthesis of the peptide either it is large peptide or the small peptide number of the techniques are available for the synthesis such as the chemical method, solution phase synthesis and others, and in such method one of the techniques is known as the solid phase peptide synthesis developed by the Robert Bruce Merrifield which completely changed and simplified the process of peptide synthesis. With the help of the SPPS up to 50 length of amino acid chain synthesis of peptides are possible [89]. In general the solid phase synthesis is defined as synthesis of the chemical compound where the reactant molecule is bounded to the solid support(such as resin) and the reactant are added in the liquid or solution phase[90].

Amino acid are added in a process which consist of the cycle which is:

- Deprotection of Amino acid
- Washing step
- Coupling of the amino acid
- Again deprotection.

This cycle continues until the desired chain or sequence is obtained for the peptide and then cleavage of the peptide from the solid support. The carboxyl group attaching to amino acid is attached to the N-terminus of developing peptide chain during peptide synthesis. This process is the opposite in synthesis of protein, in which the N-terminal of the next amino acid is

connected to the C-terminus of protein chain (N-to-C). Due to complexities of in-vitro protein synthesis, amino acid addition to the developing peptide chain occurs in a precise, step-by-step, and cyclic way. In the Solid phase peptide synthesis they all follow the same step-by-step approach of adding amino acids one at a time to the peptide chain [91]. For the solid support peptide synthesis, the following approach in the stepwise method can be helpful; each of the following steps will be briefly discussed

- The resin
- Linkers
- Coupling reagents
- Protecting group
- Cleavage reagents

Resin: For the solid support peptide synthesis, a support is required where the amino acids can bind together for peptide synthesis. Usually for the support Rink amide resins are used which is helpful in the synthesis of high-quality peptide with long sequences. These resins swell up twice the size in the SPPS solvent such as DMF. Increasing the size help in easy binding of the amines due to greater surface area. Also, this increased swelling improves solvent and reagent access to reactive sites, minimizing contaminants and by products caused by incomplete reactions and deletion sequences. The complete swelling process for dry resin can take up to 2-3 hours depending upon the resin and solvent used.

Linkers: To bind the small molecule to the polymeric resin during solid phase peptide synthesis, linker is used for covalent binding which often known as 'handle'. This linker resembles to protecting groups in solution phase synthesis and it should remain stable during the synthesis of peptide without having any affect in changing the confirmation or altering synthesis, but it must also be cleaved selectively at completion of the synthesis, and detaching the small molecule from the resin into solution. It should be noted that, as compared to solution phase synthesis, the need for a linker frequently adds two more synthetic steps to a solid phase pathway. There are many different types of linkers currently available, in which many are based on chemistry established for oligomeric solid phase peptide synthesis. The choice of the best linker for a certain type of target molecule is an important consideration when developing a solid phase synthesis.

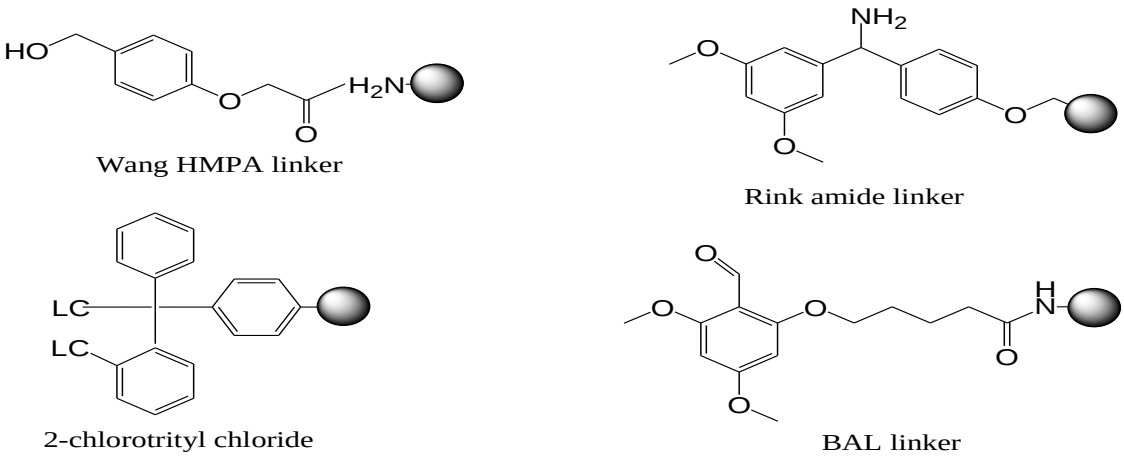


FIG3.1 : FMOC BASED SPPS LINKER

Coupling Reagents: Next step after the deprotection of the amino acid is to couple the amino acid as the N terminal is free from the protecting group and free N- terminal group can react with the upcoming amino acid Carboxylic group. These C- terminal carboxylic group requires the coupling reagents such as the Diisopropylcarbodiimide (DIC), which form a reactive intermediate compound after reacting with carboxylic group due to which it react rapidly with the deprotected N terminus of the peptide chain [92]. After the addition of new AA again the N-terminal of amino acid is protected with the temporary group and again it has to be deprotected with the appropriate chemical. This cycle of amino acid deprotection, coupling, and then again deprotection continues until desired length of the peptide sequences obtained.

Protecting group: Protecting group are those group which is attached to the N terminus of the Amino acid and help into avoiding side reactions, branching and reduction in the length during the peptide synthesis. Fmoc and Boc are two important protecting group, where the Fmoc is base labile and Boc is acid labile group.

Fmoc protecting group: Eric Atherton and Bob Sheppard developed Fmoc chemistry at the Laboratory of Molecular Biology in Cambridge in the late 1970's. One of the most common protecting groups for amino acid in the solid phase peptide synthesis is Fmoc (9-fluorenylmethoxycarbony) protection. Fmoc group attach on the N terminal of the amino acid, and it a base labile protecting group. This property of Fmoc plays an important role during the deprotection as when the base reactant is used only the Fmoc are removed and the acid labile side chain protection groups remain intact. Its chemical formula is **$C_{15}H_{11}ClO_2$** and has molar mass 258.70 g·mol−1. Fmoc group deprotection is done by using the mild Base

such as the Piperidine (PP), Piperazine (PZ). Both are mild base and they help into deprotection of amines.

Boc protecting group: Boc group also known as tert-butoxy carbonyl protecting group another amine protecting group used for the protection of N terminus of amnio acid in solid phase peptide synthesis. The protection of the amines and amino acid in Boc protection is carried out under aqueous or anhydrous solution. Boc groups is acid labile group due to which it is stable under the most nucleophiles and the bases, deprotection of Boc group is dome by using the acidic reagent such as the Trifluoracetic acid. Its chemical formula is $C_5H_9O_2$ [93].

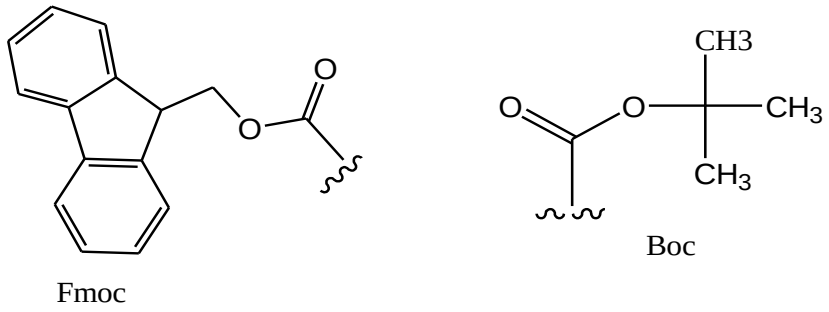


FIG3.2: Fmoc and Boc protecting Group

Cleavage of Peptide: Cleavage is last step in the Solid phase synthesis and its goal is to separate the peptide from the support and at the same time removing the remaining protecting group from the peptide. The process of the cleavage should be done quickly so that the peptide should have less exposure to the acid-based chemicals.

3.3 General method of Fmoc Protection of 3-amino benzoic acid:

- To the solution of 3-ABA (1.37g 10mmol) in 35 ml water and added sodium bicarbonate in water. Both the solution is mixed together and cooled at 5̊c (Sol A).
- Then Slowly add Fmoc-cl into the Para-dioxane. (Sol B)
- Then the Sol A is mixed into the Sol B drop by drop by consistent string in ice for 3-4 hrs.
- When both Sol A and Sol B get mixed together equal amount of distilled water is added approx. 50ml and then the aqueous layer was extracted thrice using the ethyl acetate.

- Then using the saturated solution of sodium bicarbonate, the organic layer is extracted twice.
- Then pH was maintained by using the 10% HCL and pH was checked using the pH meter.
- Again, the combined layer was extracted using the ethyl acetate thrice. And combined layer transferred to the round bottom flask (RBF).
- Then using the Rota evaporator under the reduced pressure to isolate the main compound.
- Thin layer chromatography was performed on the crude material obtained.

3.4 General method of Boc- protection of Amines:

- In the aqueous solution of 1,3 di amino propane adds sodium bicarbonate and prepare the solution A and cooled at 5℃.
- Then in Solution B add Boc Anhydride as a solution in 1,4 dioxane.
- Then using the saturated solution of sodium bicarbonate, the organic layer is extracted twice
- After the completion of the reaction add the equal amount of water 25ml and then the aqueous layer is extracted thrice with the ethyl acetate.
- After that the organic layer is extracted twice with saturated aqueous sodium bicarbonate.
- Then the pH is adjusted using the 10% HCL solution and then again extracted thrice using the DCM and the resultant layer is transferred into the round bottom flask.
- Using the Rota evaporator under the reduced pressure the main compound isolated, the crude material was used without further purification.
- Using the TLC plate at the end of reaction, compound was observed.

3.5 General method for solid phase synthesis of peptide (LP-24):

General method for the synthesis of lipopeptides are based on the solid phase peptide synthesis which is manually followed by standard Fmoc solid phase protocol using the Rink amide 4- methylbezhydrylamine salt (MBHA) resin. Loading value for the resin is 0.79mmol/g and 180mg of dry resin was weigh and swell in the solvent DMF for 3-4 hours in the vessel and then the solvent was removed by the vacuum filtration process. In each step of synthesis involves the Fmoc deprotection by using 20% piperidine in DMF and then by kaiser test using ninhydrin for deprotection test. For coupling process of Fmoc-orn (Boc)-OH onto

resin, 2eq. of DIC and HOBt in DMF was used for 4 hrs. 4 residues of Fmoc-orn (Boc)-OH was added one by one. The acetylation process was completed by adding the palmitic acid on the N terminus of peptide bound on resin using the HOBt and DIC in DMF. After the completion of the desired sequence, the lipopeptide was cleaved from the resin using the cleavage cocktail which consist 95:2.5:2.5 ratio of TFA:TIPS: Water. Using Rota evaporator and diethyl ether white precipitate obtained. Then 1:1 ratio of ACN:Water added kept in -80°c and lyophilized. Characterization of crude lipoprotein was done by HPLC using C18 column at room temperature. A linear gradient of 0-30% of sol B (0.1% TFA in ACN) and sol A (0.1% TFA in H_2O) for 35 min was used at the flow rate of 0.5ml/min. Absorbance observed at 220nm. Mass of compound was calculated by using mass spectrometry.

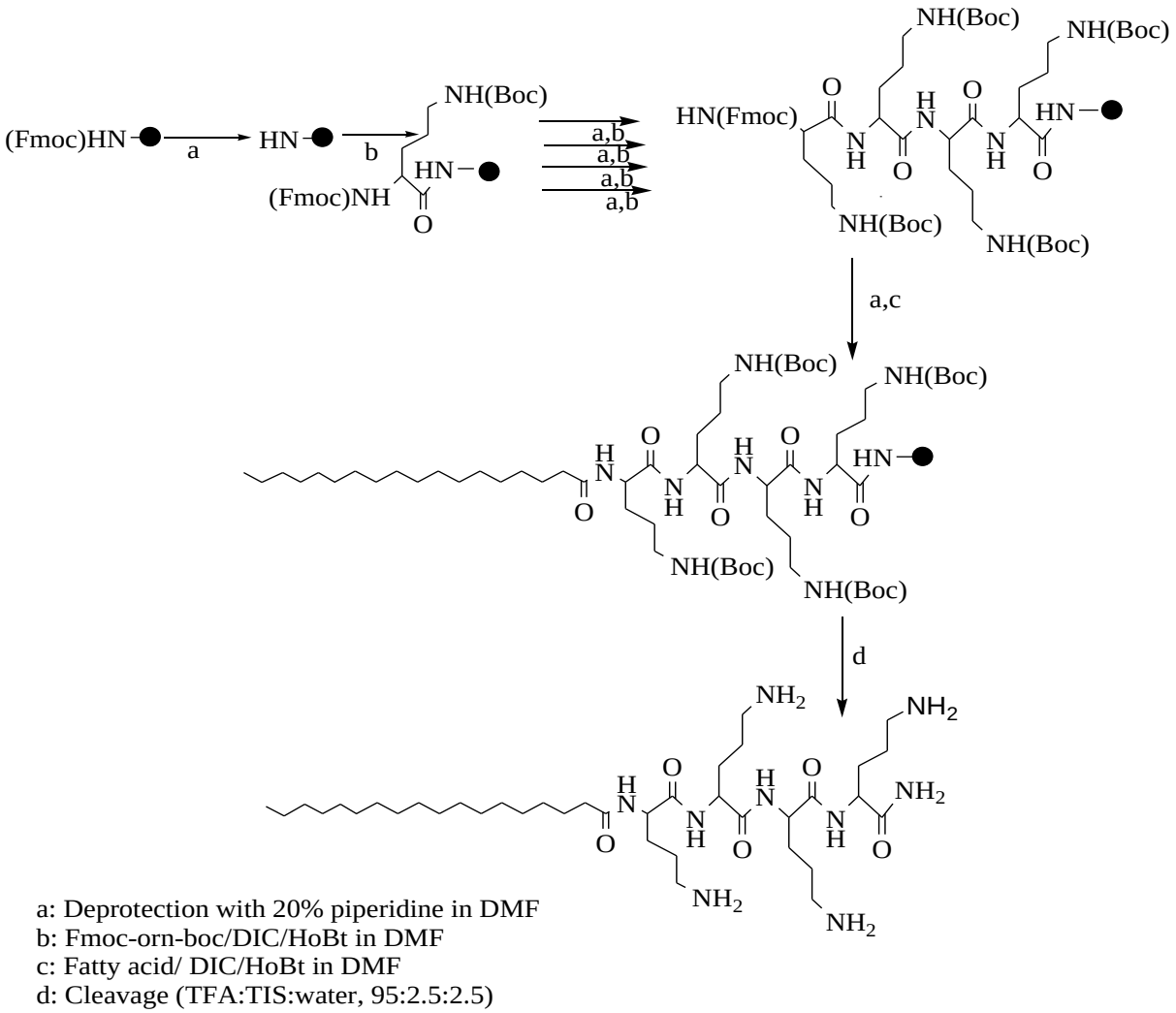


FIG 3.3 : General scheme of solid phase peptide synthesis of LP-24

3.6 Cytotoxicity Evaluation of LP24

Cytotoxicity test of LP24 was performed on the A549 cancer cell line which is a human lungs carcinogenic cell line using the Alamar Blue assay. Alamar blue assay is used to test viability of cells and also for cytotoxicity studies. It also provides few advantages over other assay such as rapid detection in toxicity of compound on cell lines, accuracy and sensitivity. In the Alamar assay Resazurin dye is present which act as active compound and help into detection of living cells. It works on the basic principle of difference between the viable and non-viable cells where in viable cell it converts into the pink or red colour. This change in the state of dye is measured by spectrophotometer by recording its absorbance between 570 and

600 nm wavelengths [94]. In this protocol activity of Lipopeptide is evaluated on Human lungs cancerous cell line A549 using Alamar blue assay.

3.6.1 Alamar assay protocol:

A549 cells were seeded in 96 micro-well plate at a cell density of 8 X 10 3 cells per100 µl. The cells were kept in an incubator with 5% CO 2 , 37°C for overnight, so that cells get adhered to the plate surface. Further, each well was administered with different concentrations of peptide (LP24) with control (cells in complete media containing FBS and DMEM). After incubation of 48h, the cells were analysed for cell viability through Alamar blue technique. For this, the cells were initially replaced with fresh medium and then 10% of resazurin (0.15mg/ml in PBS) was added to it. The wells were allowed to incubate for 2 hrs in an incubator and later the growth of the cells was measured through multi-well plate ELISA reader (Thermo Multiskan GO, Thermo Scientific, UK). The change in the absorbance was recorded at 570 nm and 600 nm respectively. Percentage cell viability was calculated against the control cells, where the non-treated cells were taken as 100% viable.

CHAPTER 4 RESULTS AND DISCUSSION

4. RESULT AND DISCUSSION:

4.1 Lipopeptide design and synthesis:

With the help of SPPS we designed 4 residue long chain of Fmoc-Orn(Boc)-OH lipopeptide with stearic acid (18-carbon chain) as a fatty acid moiety. Ornithine is a non-protein amino acid has +1 charge on each residue, due to which over all charge on peptide is +4 and Stearic acid help into providing hydrophobicity. Both Charge and hydrophobicity an important character for peptide for antiproliferative activity on cancerous cell. As charge help into binding of peptide to negatively charged surface and hydrophobicity help to penetrate peptide inside cell membrane. A balance between charge and hydrophobicity required for better antiproliferative activity of peptide [95].

In the previous study on LP24 ornithine-based peptides shows high activity against a range of clinical isolates of fungi. It was also observed that in LP24 when stearic acid fatty chain was attached than maximum antifungal potential was observed. This study shows that attaching alkyl tail increases hydrophobicity and thus increasing interaction with membrane. LP24 shows stronger antifungal activity against A. fumigatus. Charge also played an important role as discussed above and also studies shows that lipopeptide having positively charged amino acid between 4-5 shows maximum antifungal activity due to which lipopeptide synthesised for antiproliferative activity has 4 ornithine residues. But there should be charge hydrophobicity balance in lipopeptide as it was observed that when hydrophobicity was increased by adding aliphatic tail then ability to distinguish between fungal and mammalian cell was compromised at some level [96]. Thus, it appears that lipopeptides' greater hydrophobic bulk leads them toward zwitterionic membranes, which mirror Fungi and mammalian cell membranes [97].

LP24 was synthesised by solid phase peptide synthesis in which successful chain of 4 ornithine residue with stearic acid was synthesised and characterization was done through HPLC and mass spectrometry. In purification through HPLC the purity of compound was >99% and the calculated mass of compound is 439.61 and in mass spectrometry observed mass is 440.61(M+1) observed.

Compound Name	Calculated mass	Compound Sequence	Chemical formula	HPLC purity
LP24	740.61	$CH_3(CH_2)_{16}CONHnOnOnOnO\text{-}NH_2$	$C_{38}H_{77}N_9O_5$	>99%

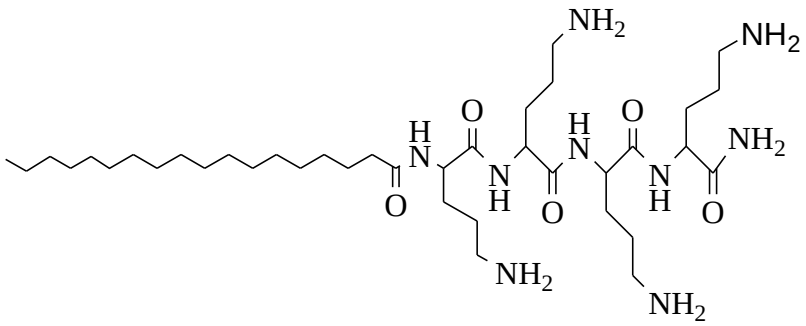


FIG 4.1: Chemical Structure of LP24

4.2 HPLC Result:

For purity of compound high performance liquid chromatography was used and absorbance was observed at 220nm.

LP24 at 220nm:

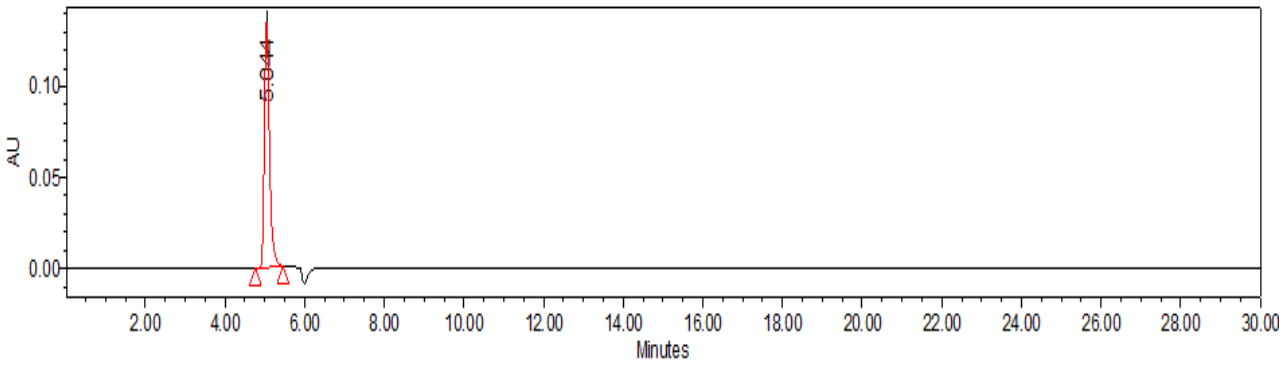


Fig 4.2 HPLC chromatogram at 220nm

Table 4.1 HPLC data for given retention time and % purity of compound.

S.No	Retentions time (min)	Area (µV*sec)	% Area	Height (µV)
1	5.044	1208991	100.00	135733

4.3. Mass Spectrometry result:

In the mass spectrometry the difference between observed and calculated mass is (M+1) which is 741.62.

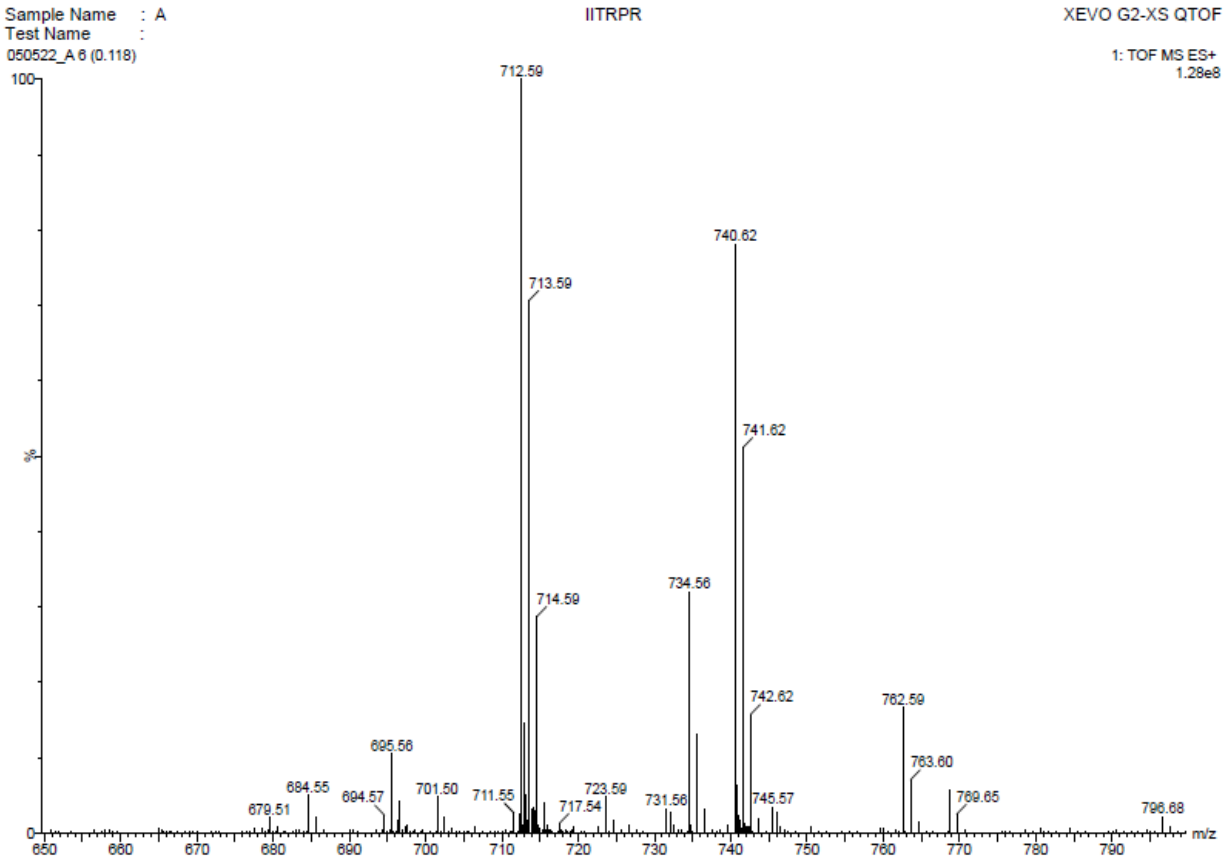


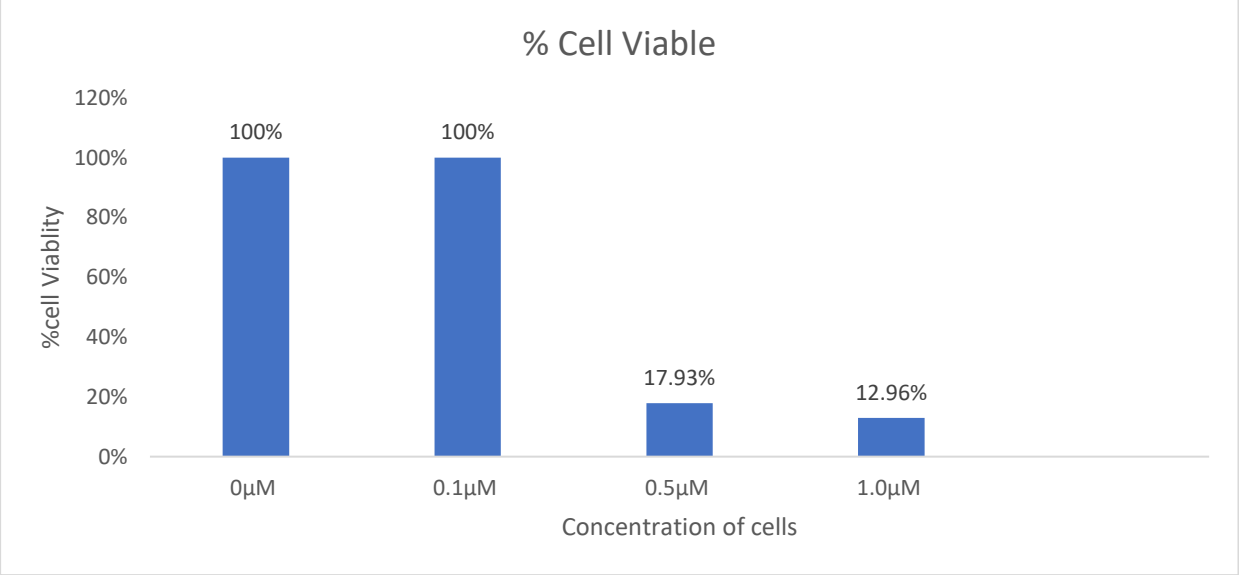
Fig 4.3 Mass spectroscopy result.

5 Cytotoxicity of LP24:

Alamar assay result of LP24 against the human lungs cancer cell line A549 showed that its selectivity toward the cancerous cell. At the initial concentration of 0 µM and 0.1 µM the viability of cells was 100% but when the concentration of peptide increased to 0.5 µM the viability remains 17.93% and at the concentration of 1.0 µM of cells total viable cells were 12.26%.the outcome of this Alamar blue assay shows that compound LP24 is toxic toward the cancerous cell and also its selectivity for cancerous cells.

Table 4.2 Different concentration of compound with % viability of cells.

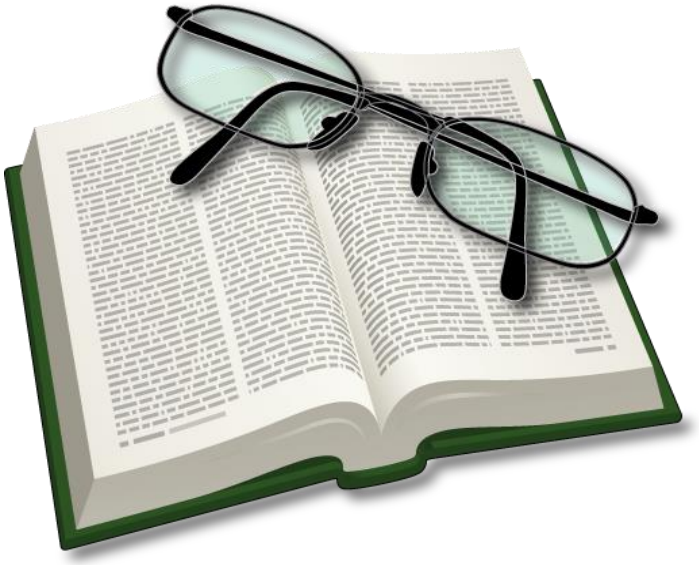
Concentration of cells	% Cell Viable
0µM	100%
0.1µM	100%
0.5µM	17.93%
1.0µM	12.96%



Interpretation:

Both results from mass spectrometry and HPLC shows the successful synthesis of lipopeptide LP24 and further results for cytotoxicity shows the compound selectivity and its anti-proliferative activity on cancer cell line A549. Further studies and comparison with other cells are ongoing for compound.

CHAPTER 5 CONLUSION



5. CONCLUSION:

In this thesis research work done describes the synthesis of short anti-cancer peptide. We successfully synthesised and designed a short compound LP24 by adding 4 residues of ornithine(non-proteogenic amino acid) and adding aliphatic chain of stearic acid to increase the hydrophobicity. Characterization of compound was done by using HPLC and Mass spectrometry. Cytotoxicity evaluation was done through Alamar blue assay on human lungs cancer cell line A549 and different concentration of lipopeptide shows different level of cytotoxicity on the cancerous cell line as at concentration of 1.0 μM more than 80% cancer cell were destroyed. This test shows the selectivity and toxic nature of compound toward the cancerous cells. Further studies on LP24 on different cell line and its affect on normal cell lines is under investigation.

CHAPTER 6 REFERENCES

REFRENCES:

[1] "What Is Cancer? - NCI," Sep. 17, 2007. https://www.cancer.gov/about-cancer/understanding/what-is-cancer (accessed May 23, 2022).

[2] H. Jayasekara, R. J. MacInnis, R. Room, and D. R. English, "Long-Term Alcohol Consumption and Breast, Upper Aero-Digestive Tract and Colorectal Cancer Risk: A Systematic Review and Meta-Analysis," *Alcohol Alcohol*, vol. 51, no. 3, pp. 315–330, May 2016, doi: 10.1093/alcalc/agv110.

[3] "Family Cancer Syndromes." https://www.cancer.org/healthy/cancer-causes/genetics/family-cancer-syndromes.html (accessed May 24, 2022).

[4] M. A. F. Hayashi, F. Ducancel, and K. Konno, "Natural Peptides with Potential Applications in Drug Development, Diagnosis, and/or Biotechnology," *Int. J. Pept.*, vol. 2012, p. 757838, 2012, doi: 10.1155/2012/757838.

[5] R. Ali, R. Rani, and S. Kumar, "New Peptide Based Therapeutic Approaches," 2013. https://www.semanticscholar.org/paper/New-Peptide-Based-Therapeutic-Approaches-Ali-Rani/7065cdd1cf4976f2a339136e9b432fcb0f6440d8 (accessed May 24, 2022).

[6] K. Kurrikoff, D. Aphkhazava, and Ü. Langel, "The future of peptides in cancer treatment," *Curr. Opin. Pharmacol.*, vol. 47, pp. 27–32, Aug. 2019, doi: 10.1016/j.coph.2019.01.008.

[7] M. Xie, D. Liu, and Y. Yang, "Anti-cancer peptides: classification, mechanism of action, reconstruction and modification," *Open Biol.*, vol. 10, no. 7, p. 200004, doi: 10.1098/rsob.200004.

[8] "Frontiers | From antimicrobial to anticancer peptides. A review | Microbiology." https://www.frontiersin.org/articles/10.3389/fmicb.2013.00294/full (accessed May 24, 2022).

[9] S. Mj, B. Ww, and B. Kw, "Cytotoxicity of rabbit macrophage peptides MCP-1 and MCP-2 for mouse tumor cells," *Antimicrob. Agents Chemother.*, vol. 28, no. 5, Nov. 1985, doi: 10.1128/AAC.28.5.626.

[10] R. J. Boohaker, M. W. Lee, P. Vishnubhotla, J. M. Perez, and A. R. Khaled, "The use of therapeutic peptides to target and to kill cancer cells," *Curr. Med. Chem.*, vol. 19, no. 22, pp. 3794–3804, 2012, doi: 10.2174/092986712801661004.

[11] "Alpha-helical cationic antimicrobial peptides: relationships of structure and function - PubMed." https://pubmed.ncbi.nlm.nih.gov/21203984/ (accessed May 24, 2022).

[12] J. C. Mai, Z. Mi, S. H. Kim, B. Ng, and P. D. Robbins, "A proapoptotic peptide for the treatment of solid tumors," *Cancer Res.*, vol. 61, no. 21, pp. 7709–7712, Nov. 2001.

[13] "Anticancer mechanisms of temporin-1CEa, an amphipathic α-helical antimicrobial peptide, in Bcap-37 human breast cancer cells - PubMed." https://pubmed.ncbi.nlm.nih.gov/23583573/ (accessed May 24, 2022).

[14] H. Suttmann *et al.*, "Antimicrobial peptides of the Cecropin-family show potent antitumor activity against bladder cancer cells," *BMC Urol.*, vol. 8, p. 5, Mar. 2008, doi: 10.1186/1471-2490-8-5.

[15] “Pardaxin-induced apoptosis enhances antitumor activity in HeLa cells - PubMed.” https://pubmed.ncbi.nlm.nih.gov/21557975/ (accessed May 24, 2022).
[16] J. J. Killion and J. D. Dunn, “Differential cytolysis of murine spleen, bone-marrow and leukemia cells by melittin reveals differences in membrane topography,” *Biochem. Biophys. Res. Commun.*, vol. 139, no. 1, pp. 222–227, Aug. 1986, doi: 10.1016/s0006-291x(86)80102-4.
[17] “BMAP-28, an Antibiotic Peptide of Innate Immunity, Induces Cell Death through Opening of the Mitochondrial Permeability Transition Pore - PMC.” https://www.ncbi.nlm.nih.gov/pmc/articles/PMC135593/ (accessed May 24, 2022).
[18] “Cell-penetrating peptides in drug development: enabling intracellular targets - PubMed.” https://pubmed.ncbi.nlm.nih.gov/17635156/ (accessed May 24, 2022).
[19] Y. Shai, Y. R. Hadari, and A. Finkels, “pH-dependent pore formation properties of pardaxin analogues.,” *J. Biol. Chem.*, vol. 266, no. 33, pp. 22346–22354, Nov. 1991, doi: 10.1016/S0021-9258(18)54578-6.
[20] Y. Shai, “Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides,” *Biochim. Biophys. Acta BBA - Biomembr.*, vol. 1462, no. 1, pp. 55–70, Dec. 1999, doi: 10.1016/S0005-2736(99)00200-X.
[21] G. Gabernet, A. T. Müller, J. A. Hiss, and G. Schneider, “Membranolytic anticancer peptides,” *MedChemComm*, vol. 7, no. 12, pp. 2232–2245, Dec. 2016, doi: 10.1039/C6MD00376A.
[22] L. Lin *et al.*, “Membrane-disruptive peptides/peptidomimetics-based therapeutics: Promising systems to combat bacteria and cancer in the drug-resistant era,” *Acta Pharm. Sin. B*, vol. 11, no. 9, pp. 2609–2644, Sep. 2021, doi: 10.1016/j.apsb.2021.07.014.
[23] J. López-Meza, A. Ochoa-Zarzosa, J. Aguilar, and P. Loeza-Lara, “Antimicrobial Peptides: Diversity and Perspectives for Their Biomedical Application,” in *Biomedical engineering, trends, research and technologies*, 2011. doi: 10.5772/13058.
[24] G. L. Bidwell and D. Raucher, “Therapeutic peptides for cancer therapy. Part I - peptide inhibitors of signal transduction cascades,” *Expert Opin. Drug Deliv.*, vol. 6, no. 10, pp. 1033–1047, Oct. 2009, doi: 10.1517/17425240903143745.
[25] L. J. Draeger and G. P. Mullen, “Interaction of the bHLH-zip domain of c-Myc with H1-type peptides. Characterization of helicity in the H1 peptides by NMR,” *J. Biol. Chem.*, vol. 269, no. 3, pp. 1785–1793, Jan. 1994.
[26] J. Lehmann *et al.*, “Antitumor activity of the antimicrobial peptide magainin II against bladder cancer cell lines,” *Eur. Urol.*, vol. 50, no. 1, pp. 141–147, Jul. 2006, doi: 10.1016/j.eururo.2005.12.043.
[27] H. S. Lee *et al.*, “Mechanism of anticancer activity of buforin IIb, a histone H2A-derived peptide,” *Cancer Lett.*, vol. 271, no. 1, pp. 47–55, Nov. 2008, doi: 10.1016/j.canlet.2008.05.041.
[28] E. D. Crawford, “Hormonal Therapy in Prostate Cancer: Historical Approaches,” *Rev. Urol.*, vol. 6, no. Suppl 7, pp. S3–S11, 2004.
[29] C. P. G, H. Takeshi, and T. Mitsuhiro, “From tumor antigens to immunotherapy,” *Int J Clin Oncol*, vol. 6, no. 4, pp. 163–170, Aug. 2001.
[30] J. Thundimadathil, “Cancer Treatment Using Peptides: Current Therapies and Future Prospects,” *J. Amino Acids*, vol. 2012, p. e967347, Dec. 2012, doi: 10.1155/2012/967347.
[31] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, and A. Jemal, “Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries,” *CA. Cancer J. Clin.*, vol. 68, no. 6, pp. 394–424, Nov. 2018, doi: 10.3322/caac.21492.

[32] Z. Cai *et al.*, “Comparative effectiveness of preoperative, postoperative and perioperative treatments for resectable gastric cancer: A network meta-analysis of the literature from the past 20 years,” *Surg. Oncol.*, vol. 27, no. 3, pp. 563–574, Sep. 2018, doi: 10.1016/j.suronc.2018.07.011.
[33] W.-D. Yu, G. Sun, J. Li, J. Xu, and X. Wang, “Mechanisms and therapeutic potentials of cancer immunotherapy in combination with radiotherapy and/or chemotherapy,” *Cancer Lett.*, vol. 452, pp. 66–70, Jun. 2019, doi: 10.1016/j.canlet.2019.02.048.
[34] “Emerging strategies in cancer therapy combining chemotherapy with immunotherapy. - Abstract - Europe PMC.” https://europepmc.org/article/med/30998963 (accessed May 27, 2022).
[35] J.-X. Di and H.-Y. Zhang, “C188-9, a small-molecule STAT3 inhibitor, exerts an antitumor effect on head and neck squamous cell carcinoma,” *Anticancer. Drugs*, vol. 30, no. 8, pp. 846–853, Sep. 2019, doi: 10.1097/CAD.0000000000000783.
[36] A. Tyagi *et al.*, “CancerPPD: a database of anticancer peptides and proteins,” *Nucleic Acids Res.*, vol. 43, no. Database issue, pp. D837-843, Jan. 2015, doi: 10.1093/nar/gku892.
[37] J. Thundimadathil, “Cancer treatment using peptides: current therapies and future prospects,” *J. Amino Acids*, vol. 2012, p. 967347, 2012, doi: 10.1155/2012/967347.
[38] D. W. Hoskin and A. Ramamoorthy, “Studies on anticancer activities of antimicrobial peptides,” *Biochim. Biophys. Acta BBA - Biomembr.*, vol. 1778, no. 2, pp. 357–375, Feb. 2008, doi: 10.1016/j.bbamem.2007.11.008.
[39] N. Papo and Y. Shai, “Host defense peptides as new weapons in cancer treatment,” *Cell. Mol. Life Sci. CMLS*, vol. 62, no. 7, pp. 784–790, Apr. 2005, doi: 10.1007/s00018-005-4560-2.
[40] T. Utsugi, A. J. Schroit, J. Connor, C. D. Bucana, and I. J. Fidler, “Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes,” *Cancer Res.*, vol. 51, no. 11, pp. 3062–3066, Jun. 1991.
[41] G. Li, Y. Huang, Q. Feng, and Y. Chen, “Tryptophan as a Probe to Study the Anticancer Mechanism of Action and Specificity of α-Helical Anticancer Peptides,” *Molecules*, vol. 19, no. 8, pp. 12224–12241, Aug. 2014, doi: 10.3390/molecules190812224.
[42] A. C. Alves, D. Ribeiro, C. Nunes, and S. Reis, “Biophysics in cancer: The relevance of drug-membrane interaction studies,” *Biochim. Biophys. Acta BBA - Biomembr.*, vol. 1858, no. 9, pp. 2231–2244, Sep. 2016, doi: 10.1016/j.bbamem.2016.06.025.
[43] “Asymmetric distribution of phospholipids in biomembranes - PubMed.” https://pubmed.ncbi.nlm.nih.gov/16880602/ (accessed May 27, 2022).
[44] G. Deliconstantinos, “Physiological aspects of membrane lipid fluidity in malignancy,” *Anticancer Res.*, vol. 7, no. 5B, pp. 1011–1021, Oct. 1987.
[45] S. Ran, A. Downes, and P. E. Thorpe, “Increased exposure of anionic phospholipids on the surface of tumor blood vessels,” *Cancer Res.*, vol. 62, no. 21, pp. 6132–6140, Nov. 2002.
[46] “Pathophysiologic Implications of Membrane Phospholipid Asymmetry in Blood Cells | Blood | American Society of Hematology.” https://ashpublications.org/blood/article/89/4/1121/139032/Pathophysiologic-Implications-of-Membrane (accessed May 27, 2022).
[47] C. Oelkrug, M. Hartke, and A. Schubert, “Mode of Action of Anticancer Peptides (ACPs) from Amphibian Origin,” *Anticancer Res.*, vol. 35, no. 2, pp. 635–643, Feb. 2015.

[48] W. Shoombuatong, N. Schaduangrat, and C. Nantasenamat, “Unraveling the bioactivity of anticancer peptides as deduced from machine learning,” *EXCLI J.*, vol. 17, pp. 734–752, 2018, doi: 10.17179/excli2018-1447.
[49] Y. Dai *et al.*, “Pro-apoptotic cationic host defense peptides rich in lysine or arginine to reverse drug resistance by disrupting tumor cell membrane,” *Amino Acids*, vol. 49, no. 9, pp. 1601–1610, Sep. 2017, doi: 10.1007/s00726-017-2453-y.
[50] P. Midoux, A. Kichler, V. Boutin, J. C. Maurizot, and M. Monsigny, “Membrane permeabilization and efficient gene transfer by a peptide containing several histidines,” *Bioconjug. Chem.*, vol. 9, no. 2, pp. 260–267, Apr. 1998, doi: 10.1021/bc9701611.
[51] O. D. K. Maddocks *et al.*, “Modulating the therapeutic response of tumours to dietary serine and glycine starvation,” *Nature*, vol. 544, no. 7650, pp. 372–376, Apr. 2017, doi: 10.1038/nature22056.
[52] K. Kawaguchi *et al.*, “Targeting methionine with oral recombinant methioninase (o-rMETase) arrests a patient-derived orthotopic xenograft (PDOX) model of BRAF-V600E mutant melanoma: implications for chronic clinical cancer therapy and prevention,” *Cell Cycle*, vol. 17, no. 3, pp. 356–361, Mar. 2018, doi: 10.1080/15384101.2017.1405195.
[53] G. Gueron *et al.*, “Game-changing restraint of Ros-damaged phenylalanine, upon tumor metastasis,” *Cell Death Dis.*, vol. 9, no. 2, p. 140, Feb. 2018, doi: 10.1038/s41419-017-0147-8.
[54] D. Bhunia *et al.*, “Spatial Position Regulates Power of Tryptophan: Discovery of a Major-Groove-Specific Nuclear-Localizing, Cell-Penetrating Tetrapeptide,” *J. Am. Chem. Soc.*, vol. 140, no. 5, pp. 1697–1714, Feb. 2018, doi: 10.1021/jacs.7b10254.
[55] V. V. Lemeshko, “Electrical potentiation of the membrane permeabilization by new peptides with anticancer properties,” *Biochim. Biophys. Acta BBA - Biomembr.*, vol. 1828, no. 3, pp. 1047–1056, Mar. 2013, doi: 10.1016/j.bbamem.2012.12.012.
[56] R. Roudi, N. L. Syn, and M. Roudbary, “Antimicrobial Peptides As Biologic and Immunotherapeutic Agents against Cancer: A Comprehensive Overview,” *Front. Immunol.*, vol. 8, 2017, Accessed: Jun. 16, 2022. [Online]. Available: https://www.frontiersin.org/article/10.3389/fimmu.2017.01320
[57] E. Oancea, M. N. Teruel, A. F. Quest, and T. Meyer, “Green fluorescent protein (GFP)-tagged cysteine-rich domains from protein kinase C as fluorescent indicators for diacylglycerol signaling in living cells,” *J. Cell Biol.*, vol. 140, no. 3, pp. 485–498, Feb. 1998, doi: 10.1083/jcb.140.3.485.
[58] O. Shamova *et al.*, “ChBac3.4: A Novel Proline-Rich Antimicrobial Peptide from Goat Leukocytes,” *Int. J. Pept. Res. Ther.*, vol. 15, no. 2, pp. 107–119, Jun. 2009, doi: 10.1007/s10989-009-9170-7.
[59] S. R. Dennison, M. Whittaker, F. Harris, and D. A. Phoenix, “Anticancer alpha-helical peptides and structure/function relationships underpinning their interactions with tumour cell membranes,” *Curr. Protein Pept. Sci.*, vol. 7, no. 6, pp. 487–499, Dec. 2006, doi: 10.2174/138920306779025611.
[60] G. Wang, X. Li, and Z. Wang, “APD3: the antimicrobial peptide database as a tool for research and education,” *Nucleic Acids Res.*, vol. 44, no. D1, pp. D1087-1093, Jan. 2016, doi: 10.1093/nar/gkv1278.
[61] M. Dathe and T. Wieprecht, “Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells,” *Biochim. Biophys. Acta BBA - Biomembr.*, vol. 1462, no. 1, pp. 71–87, Dec. 1999, doi: 10.1016/S0005-2736(99)00201-1.
[62] T. Rozek, J. H. Bowie, J. C. Wallace, and M. J. Tyler, “The antibiotic and anticancer active aurein peptides from the Australian Bell Frogs Litoria aurea and Litoria

raniformis. Part 2. Sequence determination using electrospray mass spectrometry," *Rapid Commun. Mass Spectrom. RCM*, vol. 14, no. 21, pp. 2002–2011, 2000, doi: 10.1002/1097-0231(20001115)14:21<2002::AID-RCM128>3.0.CO;2-3.
[63] D. Marsh, M. Jost, C. Peggion, and C. Toniolo, "Lipid chain-length dependence for incorporation of alamethicin in membranes: electron paramagnetic resonance studies on TOAC-spin labeled analogs," *Biophys. J.*, vol. 92, no. 11, pp. 4002–4011, Jun. 2007, doi: 10.1529/biophysj.107.104026.
[64] W. Bellamy, M. Takase, K. Yamauchi, H. Wakabayashi, K. Kawase, and M. Tomita, "Identification of the bactericidal domain of lactoferrin," *Biochim. Biophys. Acta*, vol. 1121, no. 1–2, pp. 130–136, May 1992, doi: 10.1016/0167-4838(92)90346-f.
[65] D. Gaspar, J. M. Freire, T. R. Pacheco, J. T. Barata, and M. A. R. B. Castanho, "Apoptotic human neutrophil peptide-1 anti-tumor activity revealed by cellular biomechanics," *Biochim. Biophys. Acta*, vol. 1853, no. 2, pp. 308–316, Feb. 2015, doi: 10.1016/j.bbamcr.2014.11.006.
[66] V. Ej *et al.*, "Antimicrobial and immunomodulatory activities of PR-39 derived peptides," *PloS One*, vol. 9, no. 4, Apr. 2014, doi: 10.1371/journal.pone.0095939.
[67] S. Bae *et al.*, "The effect of alloferon on the enhancement of NK cell cytotoxicity against cancer via the up-regulation of perforin/granzyme B secretion," *Immunobiology*, vol. 218, no. 8, pp. 1026–1033, Aug. 2013, doi: 10.1016/j.imbio.2012.12.002.
[68] A. Gould and J. A. Camarero, "Cyclotides: Overview and biotechnological applications," *Chembiochem Eur. J. Chem. Biol.*, vol. 18, no. 14, pp. 1350–1363, Jul. 2017, doi: 10.1002/cbic.201700153.
[69] E. Hu, D. Wang, J. Chen, and X. Tao, "Novel cyclotides from Hedyotis diffusa induce apoptosis and inhibit proliferation and migration of prostate cancer cells," *Int. J. Clin. Exp. Med.*, vol. 8, no. 3, pp. 4059–4065, Mar. 2015.
[70] Y. Lee, C. Phat, and S.-C. Hong, "Structural diversity of marine cyclic peptides and their molecular mechanisms for anticancer, antibacterial, antifungal, and other clinical applications," *Peptides*, vol. 95, pp. 94–105, Sep. 2017, doi: 10.1016/j.peptides.2017.06.002.
[71] G. Zhang *et al.*, "A novel cyclic pentapeptide, H-10, inhibits B16 cancer cell growth and induces cell apoptosis," *Oncol. Lett.*, vol. 8, no. 1, pp. 248–252, Jul. 2014, doi: 10.3892/ol.2014.2121.
[72] T. Asao, F. Takahashi, and K. Takahashi, "Resistance to molecularly targeted therapy in non-small-cell lung cancer," *Respir. Investig.*, vol. 57, no. 1, pp. 20–26, Jan. 2019, doi: 10.1016/j.resinv.2018.09.001.
[73] Z. H *et al.*, "Novel peptide myristoly-CM4 induces selective cytotoxicity in leukemia K562/MDR and Jurkat cells by necrosis and/or apoptosis pathway," *Drug Des. Devel. Ther.*, vol. 13, Jul. 2019, doi: 10.2147/DDDT.S207224.
[74] M. L. Leite, N. B. da Cunha, and F. F. Costa, "Antimicrobial peptides, nanotechnology, and natural metabolites as novel approaches for cancer treatment," *Pharmacol. Ther.*, vol. 183, pp. 160–176, Mar. 2018, doi: 10.1016/j.pharmthera.2017.10.010.
[75] S. Ay, H. Ca, and T. Y, "Zebrafish as a model to evaluate peptide-related cancer therapies," *Amino Acids*, vol. 49, no. 12, Dec. 2017, doi: 10.1007/s00726-017-2388-3.
[76] T. Sun, Y. S. Zhang, B. Pang, D. C. Hyun, M. Yang, and Y. Xia, "Engineered nanoparticles for drug delivery in cancer therapy," *Angew. Chem. Int. Ed Engl.*, vol. 53, no. 46, pp. 12320–12364, Nov. 2014, doi: 10.1002/anie.201403036.
[77] J. D. Ramsey and N. H. Flynn, "Cell-penetrating peptides transport therapeutics into cells," *Pharmacol. Ther.*, vol. 154, pp. 78–86, Oct. 2015, doi: 10.1016/j.pharmthera.2015.07.003.

[78] E. Koren and V. P. Torchilin, “Cell-penetrating peptides: breaking through to the other side,” *Trends Mol. Med.*, vol. 18, no. 7, pp. 385–393, Jul. 2012, doi: 10.1016/j.molmed.2012.04.012.
[79] E. G and L. H, “Electrically gated ionic channels in lipid bilayers,” *Q. Rev. Biophys.*, vol. 10, no. 1, Feb. 1977, doi: 10.1017/s0033583500000123.
[80] A. L. Hilchie, D. W. Hoskin, and M. R. Power Coombs, “Anticancer Activities of Natural and Synthetic Peptides,” *Adv. Exp. Med. Biol.*, vol. 1117, pp. 131–147, 2019, doi: 10.1007/978-981-13-3588-4_9.
[81] X. Hao, Q. Yan, J. Zhao, W. Wang, Y. Huang, and Y. Chen, “TAT Modification of Alpha-Helical Anticancer Peptides to Improve Specificity and Efficacy,” *PloS One*, vol. 10, no. 9, p. e0138911, 2015, doi: 10.1371/journal.pone.0138911.
[82] S. P, E. L, P. K, B. P, and A. K, “VEGF and angiopoietin signaling in tumor angiogenesis and metastasis,” *Trends Mol. Med.*, vol. 17, no. 7, Jul. 2011, doi: 10.1016/j.molmed.2011.01.015.
[83] S. K. Bhutia and T. K. Maiti, “Targeting tumors with peptides from natural sources,” *Trends Biotechnol.*, vol. 26, no. 4, pp. 210–217, Apr. 2008, doi: 10.1016/j.tibtech.2008.01.002.
[84] W. Huang *et al.*, “Learning from Host-Defense Peptides: Cationic, Amphipathic Peptoids with Potent Anticancer Activity,” *PLOS ONE*, vol. 9, no. 2, p. e90397, Feb. 2014, doi: 10.1371/journal.pone.0090397.
[85] D. Wu, Y. Gao, Y. Qi, L. Chen, Y. Ma, and Y. Li, “Peptide-based cancer therapy: Opportunity and challenge,” *Cancer Lett.*, vol. 351, no. 1, pp. 13–22, Aug. 2014, doi: 10.1016/j.canlet.2014.05.002.
[86] S. M. Farkhani, A. Valizadeh, H. Karami, S. Mohammadi, N. Sohrabi, and F. Badrzadeh, “Cell penetrating peptides: Efficient vectors for delivery of nanoparticles, nanocarriers, therapeutic and diagnostic molecules,” *Peptides*, vol. 57, pp. 78–94, Jul. 2014, doi: 10.1016/j.peptides.2014.04.015.
[87] W. Chiangjong, S. Chutipongtanate, and S. Hongeng, “Anticancer peptide: Physicochemical property, functional aspect and trend in clinical application (Review),” *Int. J. Oncol.*, vol. 57, no. 3, pp. 678–696, Jul. 2020, doi: 10.3892/ijo.2020.5099.
[88] A. A. Kaspar and J. M. Reichert, “Future directions for peptide therapeutics development,” *Drug Discov. Today*, vol. 18, no. 17–18, pp. 807–817, Sep. 2013, doi: 10.1016/j.drudis.2013.05.011.
[89] R. B. Merrifield, “Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide,” *J. Am. Chem. Soc.*, vol. 85, no. 14, pp. 2149–2154, Jul. 1963, doi: 10.1021/ja00897a025.
[90] “Solid-phase synthesis - Latest research and news | Nature.” https://www.nature.com/subjects/solid-phase-synthesis (accessed May 16, 2022).
[91] “Chemical Approaches to the Synthesis of Peptides and Proteins | Paul L.” https://www.taylorfrancis.com/books/mono/10.1201/9781003069225/chemical-approaches-synthesis-peptides-proteins-paul-lloyd-williams-fernando-albericio-ernest-giralt (accessed May 16, 2022).
[92] “Peptide Synthesis - IN.” //www.thermofisher.com/in/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/peptide-synthesis.html (accessed May 16, 2022).
[93] “N-tert-Butoxycarbonylation of Structurally Diverse Amines and Sulfamides under Water-Mediated Catalyst-Free Conditions - PMC.” https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3767329/ (accessed May 17, 2022).
[94] N. Sharma, G. Arya, R. M. Kumari, N. Gupta, and S. Nimesh, “Evaluation of Anticancer activity of Silver Nanoparticles on the A549 Human lung carcinoma cell

lines through Alamar Blue Assay," *Bio-Protoc.*, vol. 9, no. 1, p. e3131, Jan. 2019, doi: 10.21769/BioProtoc.3131.
[95] Q.-Z. Yang, C. Wang, L. Lang, Y. Zhou, H. Wang, and D.-J. Shang, "Design of potent, non-toxic anticancer peptides based on the structure of the antimicrobial peptide, temporin-1CEa," *Arch. Pharm. Res.*, vol. 36, no. 11, pp. 1302–1310, Nov. 2013, doi: 10.1007/s12272-013-0112-8.
[96] S. Lohan, J. Monga, C. S. Chauhan, and G. S. Bisht, "In Vitro and In Vivo Evaluation of Small Cationic Abiotic Lipopeptides as Novel Antifungal Agents," *Chem. Biol. Drug Des.*, vol. 86, no. 4, pp. 829–836, Oct. 2015, doi: 10.1111/cbdd.12558.
[97] S. Lohan, S. S. Cameotra, and G. S. Bisht, "Systematic study of non-natural short cationic lipopeptides as novel broad-spectrum antimicrobial agents," *Chem. Biol. Drug Des.*, vol. 82, no. 5, pp. 557–566, Nov. 2013, doi: 10.1111/cbdd.12182.