Rational Design of Derivatives of 1-N-substituted-2-N-biphenylacetyl-L(+)-isoglutamines by Synthesis and Biological evaluation as possible anticancer agents

Submitted by

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CLASS ROLL NO.: 001411402010

EXAM ROLL NO.: M4PHA1608

REGISTRATION NO: 129006 OF 2014-15

DEPARTMENT OF PHARMACEUTICAL TECHNOLOGY
JADAVPUR UNIVERSITY
Session: 2014-16

Under Guidance of
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Dedicated to
My Parents , Sisters,
Brothers,Relatives ,Friends ,
Labmates, Teachers and Sonti

Certificate of Approval

This is to certify that ***Rajesh Kumar Khatick****,* ***(Examination Roll No: M4PHA1608, Registration No: 129006 of 2014-15)*** *has carried out the research work on the subject entitled* ***"Rational Design of 1-N-substituted-2-N-(substituted biphenylacetyl)-L(+)-isoglutamines by Synthesis and Biological evaluation as possible anticancer agents"*** *under the supervision of* ***Prof. (Dr.) Tarun Jha,*** *Professor of Natural Science Laboratory, Division of Medicinal and Pharmaceutical Chemistry, Department of Pharmaceutical Technology of Jadavpur University.*

He has incorporated her findings in this thesis submitted by his in partial fulfillment of the requirements for the degree of ***Master of Pharmacy*** *(Pharmaceutical Chemistry) of Jadavpur University. He has carried out the research work independently and with proper care and attention to our entire satisfaction.*

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Declaration

I hereby declare that this thesis contains literature survey and original research work by me (Rajesh Kumar Khatick), as part of my Master of Pharmacy studies.

All informations in this document have been obtained and presented in accordance with academic rules and ethical conduct.

I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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Exam. Roll Number ***: M4PHA1608***

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Thesis Title ***: Rational Design of 1-N-substituted-2 –N-(substituted biphenylacetyl)-L(+)-isoglutamines by Synthesis and Biological evaluation as possible anticancer agents***

Signature with Date ***:***

Acknowledgement

I am highly obliged and like to express my deep gratitude and profoundness to my reverend mentor, ***Prof. (Dr.) Tarun Jha*** *,Professor of Medicinal and Pharmaceutical Chemistry Department, Jadavpur University for his excellent and constant guidance, continuous help, endless encouragement, thoughtful and freedom and stupendous co-operation throughout the project till its successful completion. I am greatly indebted to him for his motivation, fruitful suggestions and inspirations.*

I owe my deep respect to ***Prof. (Dr.) Biswajit Mukherjee****, Head of the Department of Pharmaceutical Technology, Jadavpur University for his necessary help and encouragement as the Head of this Department.*

I am thankful to all of my ***Teachers*** *in the Department of Pharmaceutical Technology, Jadavpur University for their support and help.*

I also express my sincere thanks to all the seniors of my laboratory especially to ***Mr. Amit Kumar Halder, Mr. Nilanjan Adhikari, Mr. Avinaba Mukharjee, Mr. Sourav Pahari, Miss Sanchita Dutta, Mr. Sandip Kumar Baidya*** *my labmate* ***Mr. Arpan Ghosal*** *and my junior* ***Mr. Suman Pal*** *for their valuable help.*

It is my pleasure to be thankful to ***Departmental library*** *of the Department of Pharmaceutical Technology of Jadavpur University for providing books and necessary journals.*

I am grateful to the authority ***of Jadavpur University*** *for providing all the facilities and to the* ***University Grants Commission,*** *New Delhi for financial assistance to pursue my work****.***

Finally I would like to express my deep respect to my ***parents, relatives, friends*** *for their continuous help, love and moral support throughout the period of my work.*

Date: ……

Place: Jadavpur, Kolkata *(Rajesh Kumar Khatick)*

Preface

Cancer results from a series of molecular events that fundamentally alter the normal properties of cells. In cancer cells the normal control systems that prevent cell overgrowth and the invasion of other tissues are disabled. These altered cells divide and grow in the presence of signals that normally inhibit cell growth; therefore, they no longer require special signals to induce cell growth and division.

Cancer has become most deleterious hazards among most of the people worldwide. According to the Global Cancer Report issued by the World Health Organization (WHO), there are 14.1 million new cancer cases per year worldwide and corresponding estimates for total cancer annual deaths 8.2 million from cancer disease.

The biochemical evidence suggests that glutamic acid is present in the products of hydrolysis of malignant tumours which gives the idea of glutamine antagonists as a new class anticancer agent. The fact is that the neoplastic transformation is accompanied with increase in nucleotide and protein synthesis, for which the nitrogen demand of cancerous cells increases tremendously. Glutamine promotes the hallmarks of malignancy by acting as a continuous source of energy formation, sustaining proliferative signal, enabling replicative mortality, resisting cell growth and increasing invasion and metastasis. If synthesis of glutamine can be stopped selectively in malignant cells then the uncontrolled proliferation can also be controlled. The result will be the death or inhibition of growth of cancer cells due to deficiency of required biomolecules. It is found that a number of glutamine antagonists (e.g. azaserine, DON, acivacin, chloroketone) have potent anticancer activity.

In the past, workers in our laboratory successfully developed new anticancer chemical entities, including Glutamine and Glutamamide analogues that possibly act as glutamine antagonists. In this work, the glutamine is slightly modified to its isomer, i.e., isoglutamine which is selected as lead molecule and substituted with various chemical groups in order to find a better and more active series of anticancer agents.

The thesis work includes synthesis of fourteen new 1-N-substituted-2-(substituted biphenylacetyl)-L(+) isoglutamine analogues and the evaluation of their anticancer activity by in-vitro cytotoxicity assay against human cancer cell lines as well as normal cell lines to discover more potential novel molecules for the treatment of cancer.

Content

1.CANCER

1.1.INTRODUCTION:[1,2]

Cancer is a very dangerous disease which develops when normal cells in a particular part of the body begin to grow out of control. There are different types of cancer, but they all start because of uncontrolled growth of abnormal cell, able to invade other tissues and continue to grow, divide and re-divide instead of dying and form new abnormal cells. Some types of cancer cells often travel to other parts of the body through blood circulation or lymph vessels.

There are 14.1 million new cancer cases per year worldwide and corresponding estimates for total cancer annual deaths 8.2 million from cancer disease.

1.2.HISTORY:[3,4]

1.2.1.Oldest descriptions of cancer:

Human beings and other animals have had cancer throughout the recorded history. The oldest description of cancer (although the word cancer was not used) was discovered in Egypt and dates back to about 3000 BC. It's called the Edwin Smith Papyrus and is a copy of the part of an ancient Egyptian textbook on trauma surgery. It describes 8 cases of tumors or ulcers of the breast that were removed by cauterization with a tool called the fire drill. The writing says about the disease, "There is no treatment."

Some of the earliest evidence of cancer is found among fossilized bone tumors, human mummies in ancient Egypt and in ancient manuscripts. Growths suggestive of the bone cancer called osteosarcoma have been seen in mummies. Bony skull destruction as seen in cancer of the head and neck has been found, too.

1.2.2.Origin of the word cancer:

The origin of the word cancer is credited to the Greek physician Hippocrates (460-370 BC), who is considered the "Father of Medicine." Hippocrates used the terms carcinos and carcinoma to describe non-ulcer forming and ulcer-forming tumors. In Greek, these words refer to a crab, most likely applied to the disease because the finger-like spreading projections from a cancer called to mind the shape of a crab. The Roman physician, Celsus (28-50 BC), later translated the Greek term into cancer, the Latin word for crab. Galen (130-200 AD), another Greek physician, used the word oncos (Greek for swelling) to describe tumors. Although the crab analogy of

Hippocrates and Celsus is still used to describe malignant tumors, Galen's term is now used as a part of the name for cancer as well as its specialists – oncology and oncologists.

1.2.3.Cancer in the sixteenth to eighteenth centuries:

During the Renaissance, beginning in the 15th century, scientists developed greater understanding of the human body. Scientists like Galileo and Newton began to use the scientific method which later was used to study diseases. Autopsies, done by Harvey (1628), led to an understanding of the circulation of blood through the heart and body that had until then been a mystery.

In 1761, Giovanni Morgagni of Padua was the first to do something which has become routine today – he did autopsies to relate the patient's illness to pathologic as findings after death. This laid the foundation for scientific oncology the study of cancer.

The famous Scottish surgeon John Hunter (1728-1793) suggested that some cancers might be cured by surgery and described how the surgeon might decide which cancers to operate on. If the tumor had not invaded nearby tissue and was "moveable," he said, "There is no impropriety in removing it."

A century later the development of anesthesia allowed surgery to flourish and classic cancer operations such as the radical mastectomy i,e., removal of breast were developed.

1.2.4.Cancer in the nineteenth century:

The 19th century saw the birth of scientific oncology with use of the modern microscope in studying diseased tissues. Rudolf Virchow, often called the founder of cellular pathology, provide the scientific basis for the modern pathologic as study of cancer. As Morgagni had linked autopsy findings seen with the unaided eye with the clinical course of illness, so Virchow correlated microscopic pathology to illness.

This method not only allowed a better understanding of the damage cancer had done but also aided the development of cancer surgery. Body tissues removed by the surgeon could now be examined and a precise diagnosis could be made. The pathologist could also tell the surgeon whether the operation had completely removed the cancer or not.

1.3.STATISTICS:

1.3.1.World Wide Cancer:[5,6,7]

The statistics about cancer incidence and mortility for cancer world wide are available from The International Agency for Research on Cancer's GLOBOCAN database and The world Health Organisation (WHO)'s Global Health Observatory as well as The United Nations (UN)'s World Population Prospects reports.

All Cancers (excluding non-melanoma skin cancer) Estimated Incidence, Mortality and Prevalence Worldwide in 2012

Estimated numbers (thousands)	Men			Women			Both sexes		
	Cases	Deaths	5-year prev.	Cases	Deaths	5-year prev.	Cases	Deaths	5-year prev.
World	7410	4653	15296	6658	3548	17159	14068	8202	32455
More developed regions	3227	1592	8550	2827	1287	8274	6054	2878	16823
Less developed regions	4184	3062	6747	3831	2261	8885	8014	5323	15632
WHO Africa region (AFRO)	265	205	468	381	250	895	645	456	1363
WHO Americas region (PAHO)	1454	677	3843	1429	618	4115	2882	1295	7958
WHO East Mediterranean region (EMRO)	263	191	461	293	176	733	555	367	1194
WHO Europe region (EURO)	1970	1081	4791	1744	852	4910	3715	1933	9701
WHO South-East Asia region (SEARO)	816	616	1237	908	555	2041	1724	1171	3278
WHO Western Pacific region (WPRO)	2642	1882	4493	1902	1096	4464	4543	2978	8956
IARC membership (24 countries)	3689	1900	9193	3349	1570	9402	7038	3470	18595
United States of America	825	324	2402	779	293	2373	1604	617	4775
China	1823	1429	2496	1243	776	2549	3065	2206	5045
India	477	357	665	537	326	1126	1015	683	1790
European Union (EU-28)	1430	716	3693	1206	561	3464	2635	1276	7157

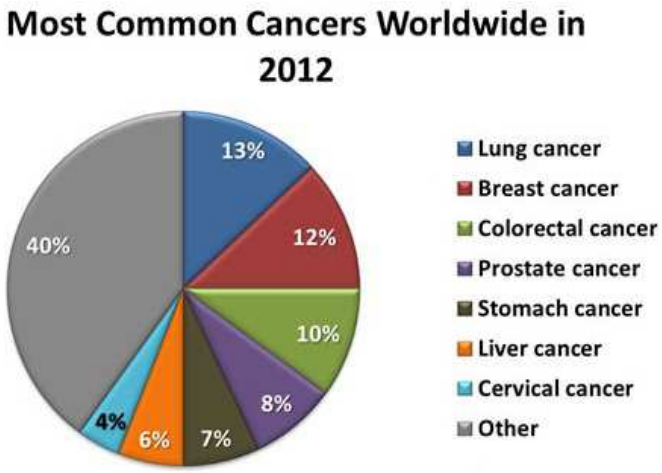
Cancer Cases-

In 2012, the most common cancers worldwide (for both sexes) were—

1. Lung cancer (13% of all cancers diagnosed; 1.8 million people).
2. Breast cancer (12% of all cancers diagnosed; 1.7 million people).
3. Colorectal cancer (10% of all cancers diagnosed; 1.4 million people).
4. Prostate cancer (8% of all cancers diagnosed; 1.1 million people).
5. Stomach cancer (7% of all cancers diagnosed; 952,000 people).
6. Liver cancer (6% of all cancers diagnosed; 782,000 people).
7. Cervical cancer (4% of all cancers diagnosed; 528,000 people).

In 2012, cancers worldwide for males and females were—

- Among males: Lung, prostate, colorectal, stomach, and liver.
- Among females: Breast, colorectal, lung, cervical, and stomach.

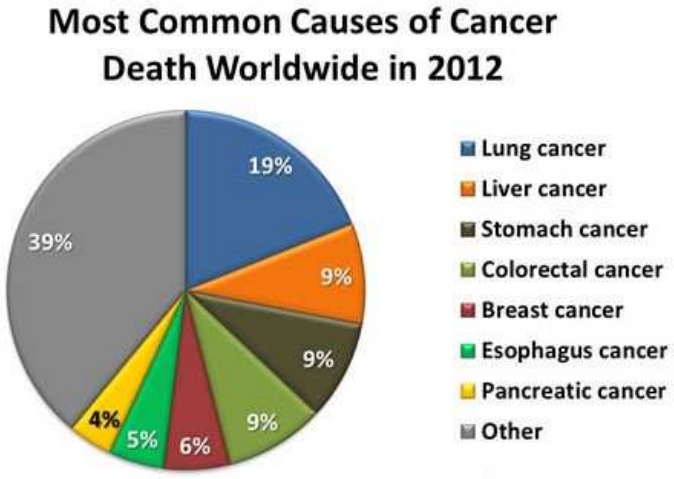


Cancer Deaths-

An estimated 168.1 million years of healthy life are lost due to cancer every year.

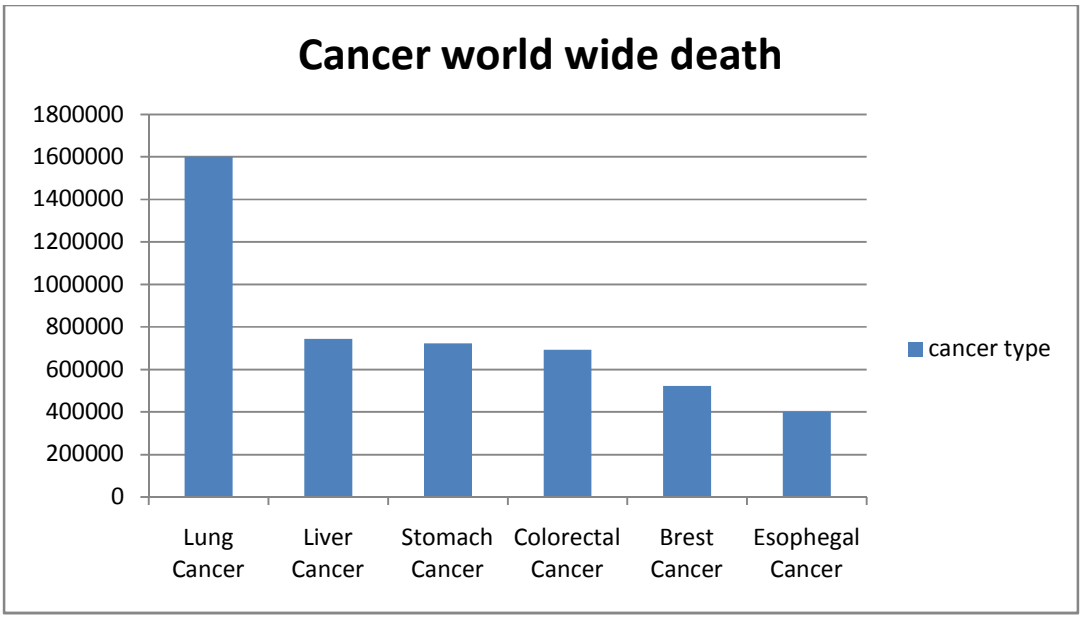
In 2012, the most common causes of cancer death worldwide (for both sexes) were—

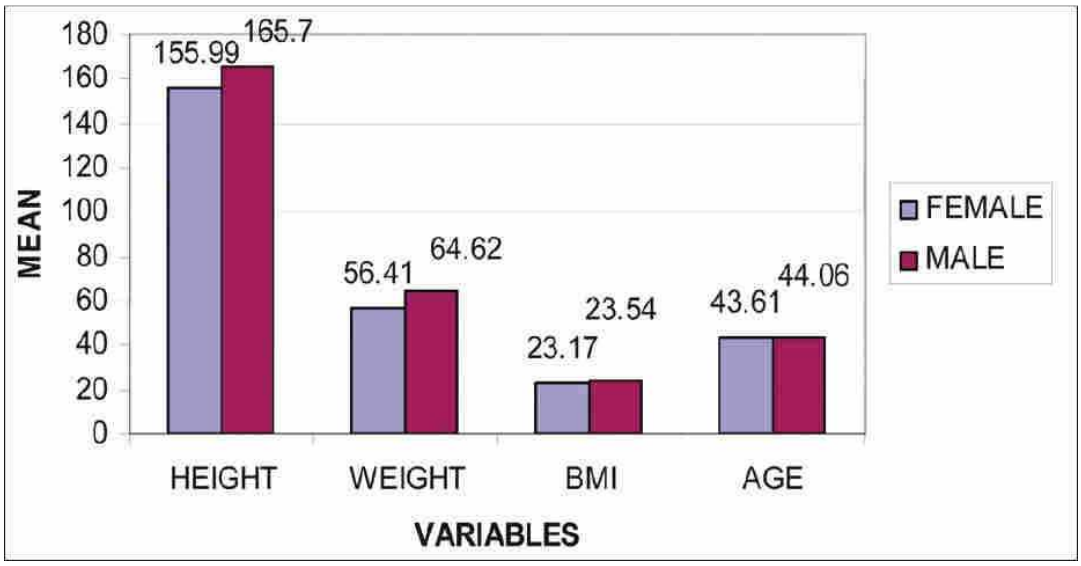
1. Lung cancer (19% of all cancer deaths; 1.6 million people).
2. Liver cancer (9% of all cancer deaths; 745,000 people).
3. Stomach cancer (9% of all cancer deaths; 723,000 people).
4. Colorectal cancer (9% of all cancer deaths; 694,000 people).
5. Breast cancer (6% of all cancer deaths; 522,000 people).
6. Cancer of the esophagus (5% of all cancers diagnosed; 400,000 people).
7. Pancreas cancer (4% of all cancers diagnosed; 330,000 people).



In 2012, the most common causes of cancer death worldwide (for males and females) were—

- Among males: Lung, liver, stomach, colorectal, and prostate.
- Among females: Breast, lung, colorectal, cervical, and stomach.





1.3.2.Tobacco, the case for primary prevention: Tobacco consumption remains the most important avoidable cancer risk. In the 20th century, approximately 100 million people died world-wide from tobacco-associated diseases (cancer, chronic lung disease, cardiovascular disease and stroke). Half of regular smokers are killed by the habit. One quarter of smokers will die prematurely during middle age (35 to 69 years). The lung cancer risk for regular smokers as compared to non-smokers (relative risk, RR) is between 20 and 30 fold. Roughly 90 per cent of lung cancers in both men and women are attributable to cigarette smoking. For bladder and renal pelvis, the RR is five-six but this means that more than 50 per cent of cases are caused by smoking. Involuntary (passive) tobacco smoke is carcinogenic and may increase the lung cancer risk by 20 per cent.

1.3.3.Infection and cancer: intervention is key: In developing countries, up to 23 per cent of malignancies are caused by infectious agents, including hepatitis B and C virus (liver cancer), human papilloma viruses (cervical and ano-genital cancers), and Helicobacter pylori (stomach cancer). In developed countries, cancers caused by chronic infections only amount to approximately 8 per cent of all malignancies. Today, more than 80 per cent of all cervical cancer deaths occur in developing countries.

1.4.CANCER SCENARIO IN INDIA: [2,8,9,10,11]

The current Indian population is 1,270,272,105(1.27 billion). The incidence of cancer in India is 7090 per 100,000 population and cancer prevalence is established to be around 2,500,000 (2.5 million) with over 800,000 new cases and 5,50,000 deaths occurring each year. More than 70% of the cases present in advanced stage accounting for poor survival and high mortality. About 6% of all deaths in India are due to cancers which contribute to 8% of global cancer mortality.

India has a National Cancer Control Programme which was established in 1975–76. This has contributed to the development of Regional Cancer Centres (RCCs), oncology wings in medical colleges and support for purchase of teletherapy machines. The District Cancer Control Programme was initiated but did not result in sustainable and productive activity. Leading cancer sites in various cancer registry areas are shown in Figure1.1

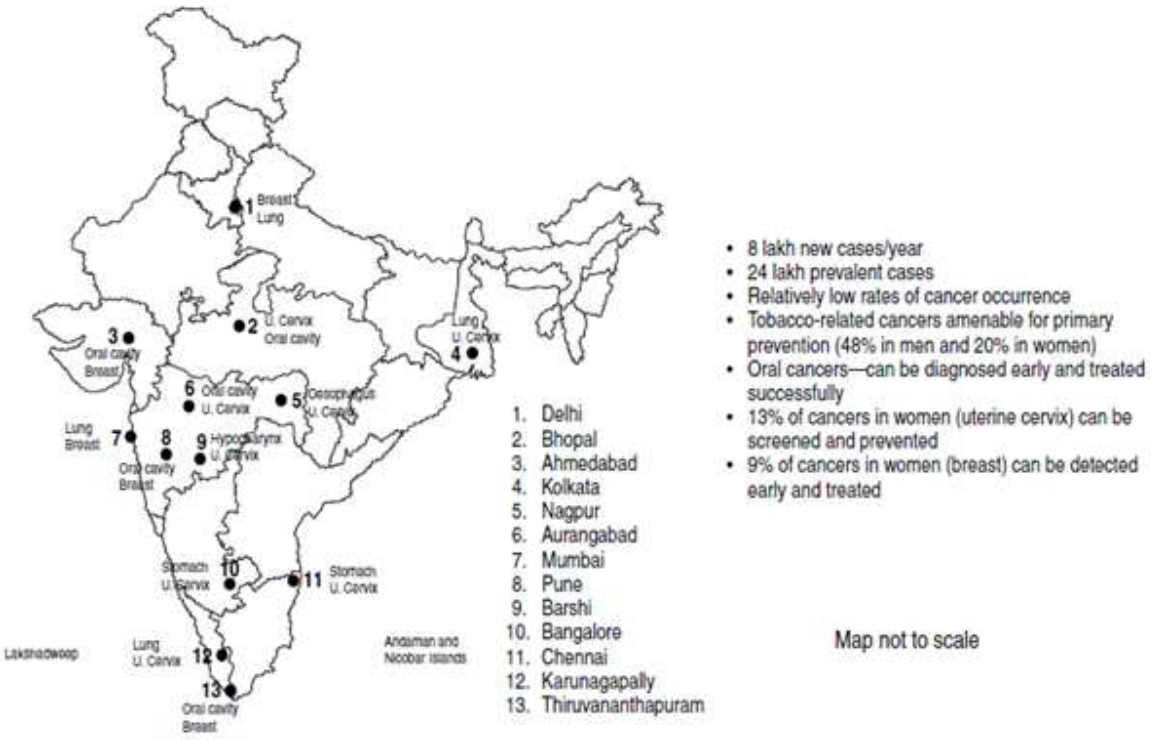


Figure 1.1 India:pattern

Epidemiologic Trends:

According to Indian Council of Medical Research (ICMR) data[8] on site specific cancer burden in males, the most common are cancers of mouth/pharynx, esophagus, stomach, lung/bronchi while as in females, the common cancers are cervix, breast, mouth/oropharynx and esophagus.

Carcinoma breast is more common in urban females. Breast cancer is the leading cancer among females as reported in registries from Mumbai, Delhi and Bangalore while in the rest of registries, cancer cervix is the leading cancer followed by breast cancer. The estimated number of breast cancer cases in India[2,9] for the year 2010, 2015 and 2020 will be approximately 90,659; 1,06,124 and 1,23,634 respectively. So breast cancer is expected to cross the figure of 1,00,000 cases in year 2015.

The annual global incidence of carcinoma cervix is approximately 5,00,000 cases and India contributes about onefifth of the burden ,ie,1,00,000 cases annually. In South India, cancer cervix is the most common cancer among females. The incidence of cancer cervix in Chennai is 99 per 1,00,000. Over the years in spite of decreasing incidence of cervical cancers, the gynaecologic cancers have increased in India and are contributing about 30% of total cancers among women in India. Among these, carcinoma cervix followed by carcinoma ovary and corpus uteri are the major contributors.

The estimated number of cancer cases related to digestive system were 107,030 in males and 86,606 in females for the year 2010. The major three cancers contributing were stomach cancers (19.8%), esophagus (18.6%) and colon cancers (14.2%). Esophageal cancers are reported maximum from South India (Karnataka, Tamil Nadu) and also from states of Maharashtra, Gujarat, Jammu & Kashmir and parts of Northeastern states. Age adjusted incidence of esophageal cancers in females in Bangalore is one of the highest in the world (8.3/1,00,000). Carcinoma stomach has highest incidence from South India especially Chennai and Bangalore.

Age adjusted incidence of gall bladder cancers in Delhi is one of the highest in the world (8.9/1,00,000). Also gall bladder cancers are reported in increasing incidence from North Indian states and West Bengal.

Carcinoma lung is having highest incidence from Mumbai, Delhi, Bhopal regestries and hypopharyngeal and penile cancers were more reported in Baarshi registry.

Overall high incidence of oral cavity cancers has been reported from Ahmedabad while as tongue cancers were reported more in Bhopal (8.8/100,000). Cancers of oral cavity, tongue and laryngeal cancers contribute maximum towards head and neck cancers.

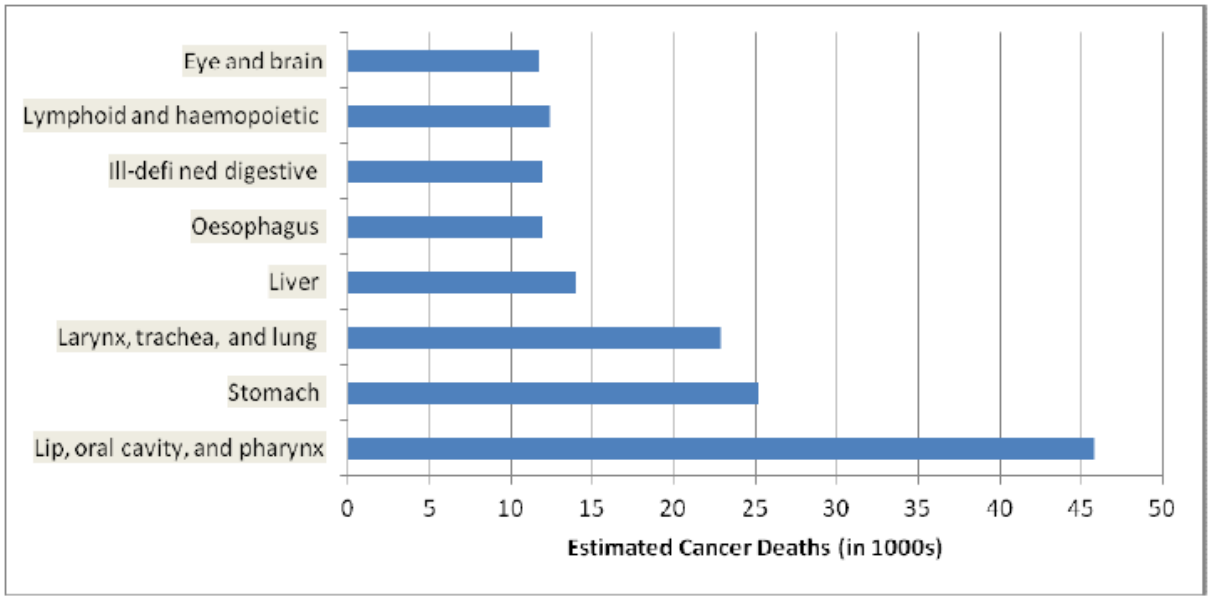
India is a culturally diverse country with huge regional and rural-to-urban variations in lifestyles and in age-specific adult death rates. A recent study[10] found large variations in the type of cancer in various regions and subpopulations of India. It concluded that:

• More than 70% of fatal cancers occur in productive ages of 30–69 years.

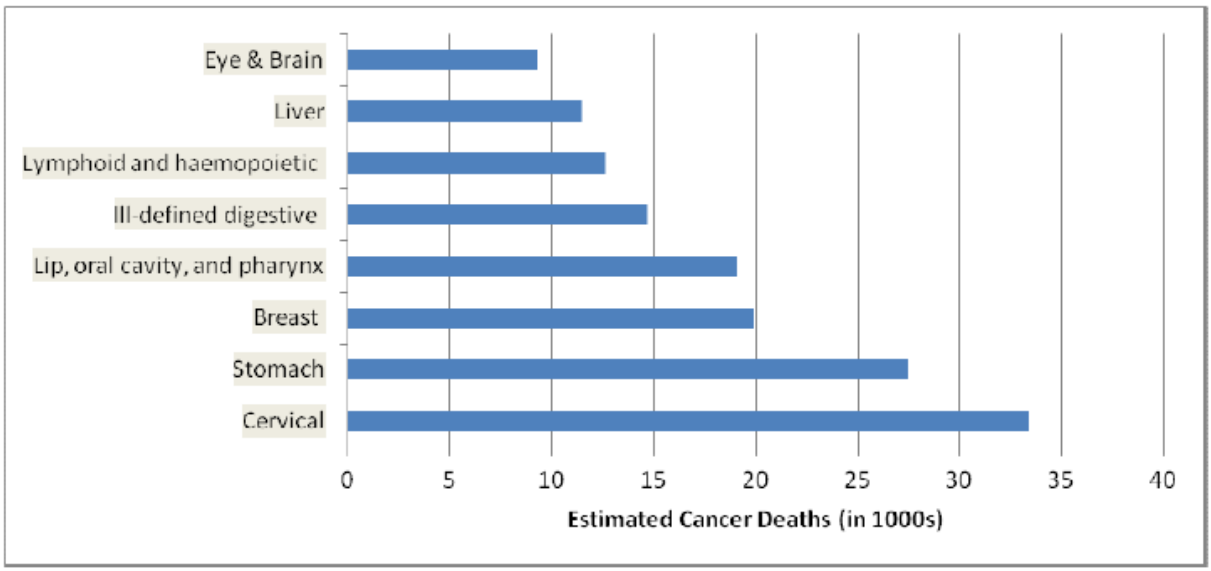
• Rates of cancer deaths were generally similar between rural and urban areas and about twice as high in the least versus the most educated.

• A large proportion of cancer deaths in middle age (30–69 years) arise from tobacco-related cancers, particularly in the northeastern states of India Lip ,Oral cavity & Pharynx Cancer in Men & Cervical Cancer in woman is the most common cancer responsible for the death in Indians based on the study conducted & funded by Bill & Melinda Gates foundation & US National Institute of Health[7].

Graph 1:- Estimated rates of cancer mortality (1000s) for main cancer aged 30–69 years (Men)



Graph 2:- Estimated rates of cancer mortality (1000s) for main cancer aged 30–69 years (Women)



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2.MATRIX METALLOPROTEINASES

2.1. INTRODUCTION

Matrix Metalloproteinases (MMPs) are universal expressed metzicin super family of multidomain calcium dependent zinc containing endopeptidases are the proteolytic peptidases that breaks peptide bonds of nonterminal amino acids i.e within the molecules responsible for the tissue remodelling and degradation of the extracellular matrix (ECM), and connective tissue such as collagens, elastins, gelatin, matrix glycoproteins, and proteoglycan. MMPs are usually minimally expressed in normal physiological conditions such as homeostasis is maintained. However, MMPs are regulated by hormones, growth factors, and cytokines, and are involved in ovarian functions. Endogenous MMP inhibitors (MMPIs) and tissue inhibitors of MMPs (TIMPs) strictly control these enzymes. Over-expression of MMPs results in an imbalance between the activity of MMPs and TIMPs that can lead to a variety of pathological disorders.[1–5]

The extracellular matrix (ECM) is primarily collection of extracellular protein molecules secreted by local cells that provides structural and biochemical support to the surrounding cells which are embedded in a hydrated polysaccharide gel. The animal extracellular matrix contain the interstitial matrix and the basement membrane. Interstitial matrix is present between various animal cells (i.e., in the intercellular spaces). The polysaccharides gel and fibrous protein molecules fill the interstitial space and act as a compression buffer against the stress placed on the ECM. The composition of ECM varies between multicellular structures; however, cell adhesion, cell-to-cell communication and differentiation are common functions of the ECM because multicellularity evolved independently in different multicellular lineages (e.g.,osteoblasts, chondrocytes).[6-9]

All MMPs family members are secreted as inactive proenzymes (zymogens) and are thought to be activated in the tissue by cleavage of the propeptide. All MMPs contain Zn^{2+} at the catalytic site and, in addition, require Ca^{2+} for stability and activity. The metzincin super family is distinguished by a highly conserved motif containing 3-histidine that bind to zinc at the catalytic site and a conserved methionine that sits beneath the active site.[10] MMPs are to be involved in highly specific cleavage of cell surface receptors, the release of apoptotic ligands (such as FAS ligand) and chemokine or cytokine inactivation.[11] MMPs also plays a major role on

cell behaviors such as cell proliferation , migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis and host defence.[12] MMPs also called Matrixin, the matrixin activities are also regulated by activation of the precursor zymogens and inhibition by endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs), an imbalance between the activity of MMPs and TIMPs can cause pathological disorders.

Matrix metalloproteinases are excreted by a variety of connective tissue and pro-inflammatory cells including fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils, and lymphocytes. These enzymes are expressed as zymogens, which are subsequently processed by other proteolytic enzymes (such as serine proteases, furin, plasmin, and others) to generate the active forms. Under normal physiological conditions, the proteolytic activity of the MMPs is controlled at any of the following three known stages: activation of the zymogens, transcription, and inhibition of the active forms by various tissue inhibitors of MMPs (TIMPs). In pathological conditions this equilibrium is shifted toward increased MMP activity leading to tissue degradation.

2.2. HISTORY

MMPs were initially discovered, in 1962, by Jerome Gross and Charles M. Lapiere while studying an active enzyme in the culture media of tissue fragments of the degradation of triple-helix of native type I collagen observed during the metamorphosis of a tadpole tail fin skin.(a tadpole tail by placing in a collagen matrix plate)[13] Thus the enzyme was named as interstitial collagenase. Then, In 1968 this enzyme was first isolated from human skin in the inactive form, proMMP (also called MMP zymogen).[14] It was later found in both invertebrates and plants.[15] They are distinguished from other endopeptidases by their dependence on metal ions as cofactors, their ability to degrade extracellular matrix (ECM), and their specific evolutionary DNA sequence. Plant MMPs are conserved proteolytic enzymes found in a wide range of monocotyledonous and dicotyledonous plant species. They play crucial roles in many aspects of plant physiology including growth, development, and the response to stress such as pathogen attack.[16] In 1990, “cysteine switch” was discovered which described the cysteine switch mechanism was responsible for regulating the enzyme in its inactive form.[17] After the complete sequencing of the human genome, it was determined that twenty four different genes encoded a set of all human MMPs.[18] Matrixins are also found in Arabidopsis,[19] Hydra,[20] and Sea urchin.[21]

2.3. STRUCTURE

MMPs are homologous proteins, which can be classified into six categories according to substrate recognition and cleavage mechanism: collagenases, stromelysins, matrilysins, gelatinases, membrane-associated MMPs and MMPs with no group designation. X-ray crystallography and nuclear magnetic resonance (NMR) studies have made it possible to determine the structures of many MMPs.[22] MMPs are zinc and calcium-dependent endopeptidases, which are synthesized from inactive proMMPs. Commonly secreted from cells in its inactive form, with the exception of membrane- associated MMPs (MT-MMPs), this prevents MMPs from cleaving essential components in cells. The enzyme is divided into three domains: N-terminal propeptide, catalytic domain and C-terminal domain (Figure 2.1)[23]

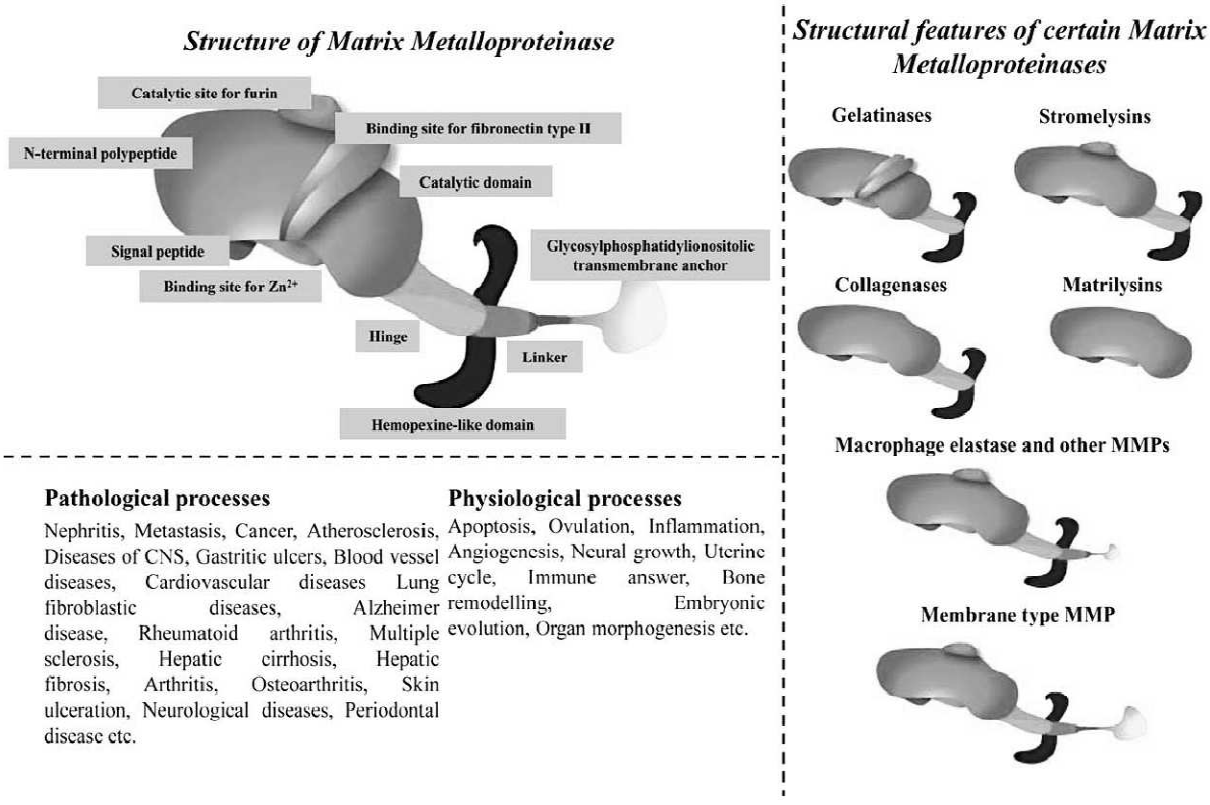

Figure 2.1. Specific structural features of certain MMPs groups (right). MMPs domain structures and their roles in physiological and pathophysiological processes (left).

2.3.1. N-Terminal Propeptide

Containing approximately 80 amino acids, N-terminal propeptide ensures enzyme latency. This domain has to be proteolytically cleaved in order to make the enzyme active. The MMPs are initially synthesized as inactive zymogens with a pro-peptide domain that must be removed before the enzyme is active. The pro-peptide domain is part of the "cysteine switch." This contains a conserved cysteine residue that interacts with the zinc in the active site and prevents binding and cleavage of the substrate, keeping the enzyme in an inactive form. In the majority of the MMPs, the cysteine residue is in the conserved sequence PRCGxPD (Pro-Arg-Gly-Cys-X-Pro-Asp where X represents any amino acid) is present,Cleavage of the propeptide triggers proMMP activation. Some MMPs have a prohormone convertase cleavage site (Furin-like) as part of this domain which are activates the enzyme serine proteinases such as plasmin when cleaved.

Activation of MMPs by cystein switch mechanism is shown in Figure 2.2.

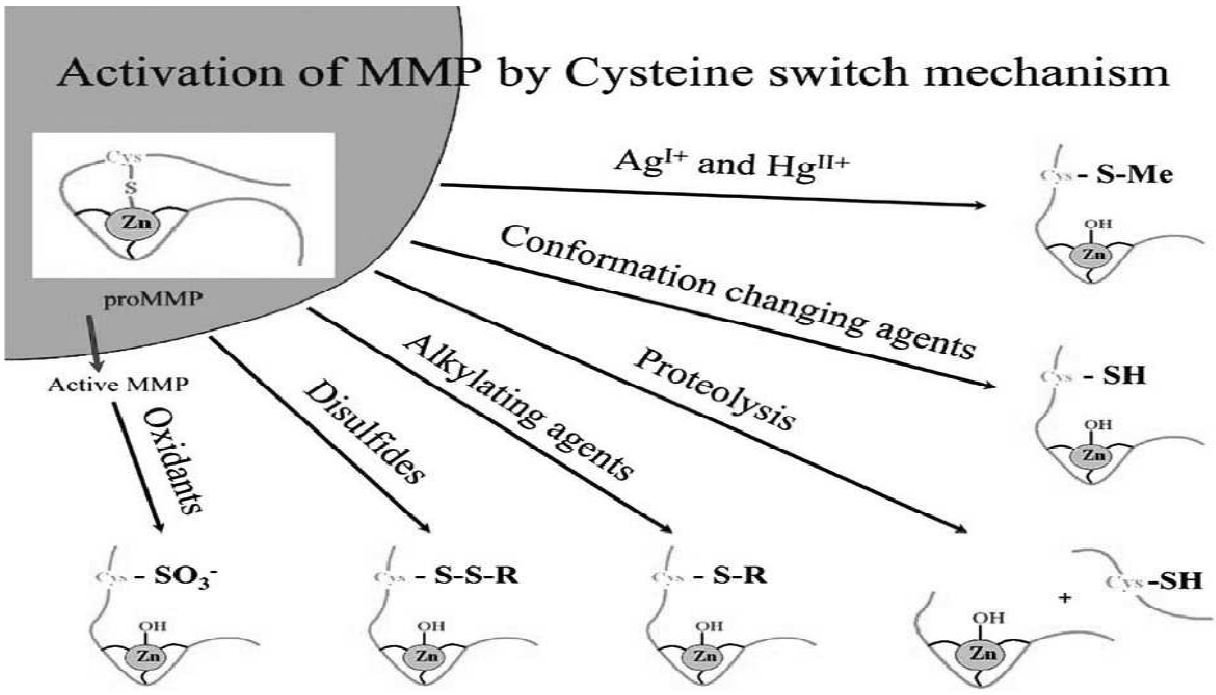

Figure 2.2. Activation of MMPs by cysteine switch mechanism [74]. Activation of MMPs through cysteine switch can be achieved by several ways i) treatment with oxidants, ii) disulfides and iii) alkylating agents, iv) proteolytic cleavage, v) usage of agents changing conformation, and vi) silver(I) and mercury(II) ions.

2.3.2. Catalytic Domain

X-ray crystallographic structures of several MMP catalytic domains have shown that this domain is an oblate sphere measuring 35 x 30 x 30 Å (3.5 x 3 x 3 nm). The active site is a 20 Å (2 nm) groove that runs across the catalytic domain.

The catalytic domain consists of five β-sheets, three α-helices and connecting loops. It is composed of 170 amino acids and contains zinc-binding motif (His-Glu-His-XX-XXXX-Gly-His, where X represents any amino acid) associated with methionine, which forms a unique structure known as the methionine loop. The catalytic domain contains two zinc(II) ions and two or three calcium(II) ions. The first Zn^{2+} ion present in the active site directly participates in catalytic processes. The second Zn^{2+} ion (also called structural) and Ca^{2+} ions are approximately 12 nm far from the Zn^{2+} ion in the catalytic site. Calcium ions are necessary to stabilize the domain structure [24].

2.3.3. The hinge region

A flexible hinge or linker region which connects the catalytic domain to the C-terminal domain. Although this 75 amino acids long region and has no determinable structure, it is very important for the enzyme's stability.

2.3.4. The hemopexin-like C-terminal domain

The C-terminal domain (or hemopexin-like domain) is structurally similar to serum proteins of the hemopexin family. The domain has a relatively large surface area for protein-protein interactions e.g. cell membrane receptors. It is ellipsoid shaped with propeller-like sub domain, The β sheets or blades arrange themselves symmetrically around a central channel, resulting into a four-bladed β-propeller structure,where each leaf of the "propeller" is composed of 4 antiparallel β-sheets and one α- helix. The first and fourth leaf are linked by a disulfide bridge. The flat surface provided by this structure is believed to be involved in interactions between proteins and is a determinant of substrate specificity, for example TIMP interacts with this site. However, this domain is not present in plants and nematodes.

ADAM (a disintegrin and metalloproteinase) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) are the two families of metzincin proteinases

that are closely related to the MMPs. Mostly membrane-anchored and pericellular space functioning ADAMs play roles in fertilization, development, and cancer . ADAMs perform their function in a nonproteolytic manner. The generally secreted and soluble ADAMTS enzymes, function during ECM assembly, ovulation, and cancer and have a protease, a disintegrin, and one or more thrombospondin domains [25].

MMPs are believed to remodel the ECM as they are capable of degrading ECM molecules. Likewise, MMPs may carry out significant functions during embryonic development as ECM remodelling is considered a critical part of tissue growth and morphogenesis. Additionally, MMPs also influence many cellular functions during development and normal physiology. For example(Figure 2.3)

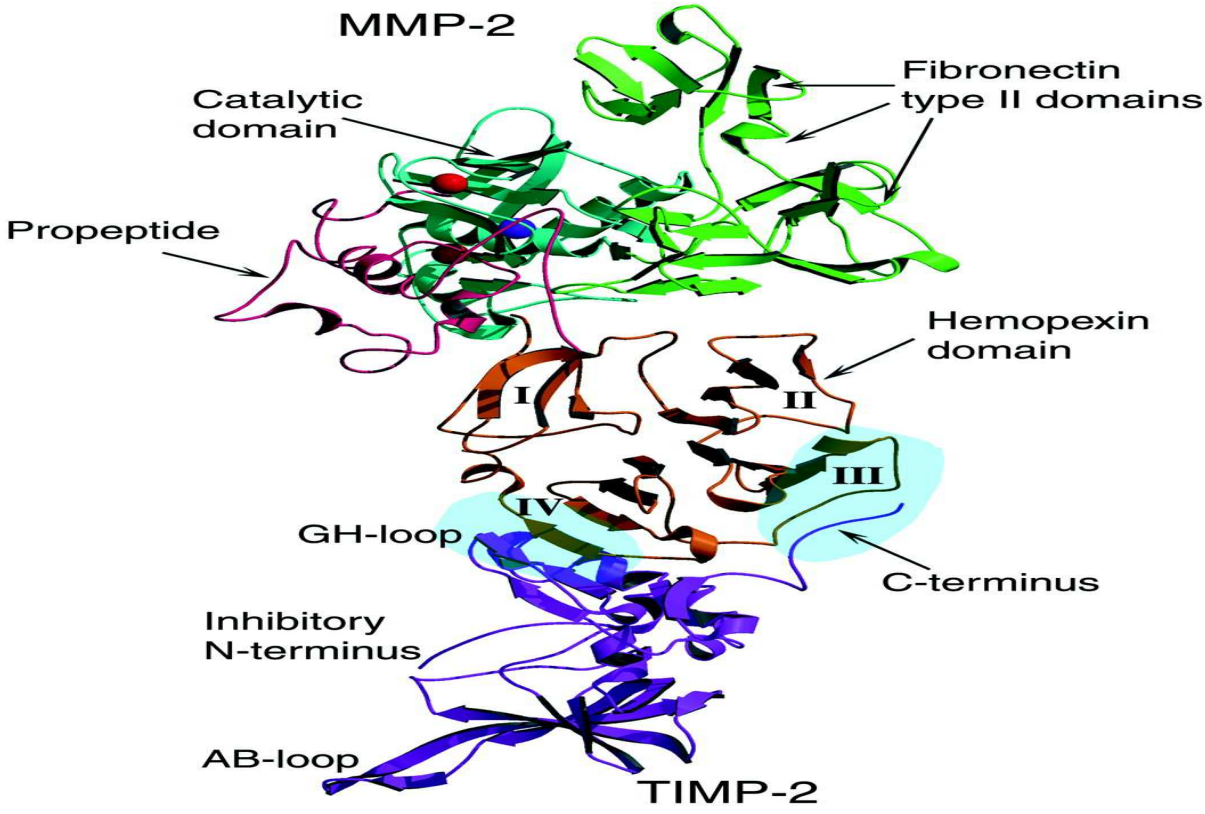

Figure 2.3. Structure of the proMMP-2/TIMP-2 complex. Overall conformation: the proteinase and inhibitor interact via their C-terminal domains. The catalytic site of MMP-2 and the inhibitory active site of TIMP-2 are turned away from each other. This topology excludes an inhibitory interaction between the proteinase and inhibitor and implies that both proteins remain

fully functional in the complex. Catalytic and structural Zn2+ ions are colored red and Ca2+ ion purple. The β-propeller blades of the hemopexin domain are numbered from I to IV. Two light blue ellipsoids in blades III and IV indicate two areas of interaction between proMMP-2 and TIMP-2 molecules.

2.4. MODE OF ACTION OF METRIX METALLOPROTEINASES

- Allowing cell migration through degradation of ECM molecules.
- Altering cellular behavior by changing ECM micro-environment.
- Modulating the activity of biologically active molecules by direct cleavage, release from bound stores, or the modulating of the activity of their inhibitors.

MMPs may effect cell migration is shown in Figure 2. 4.

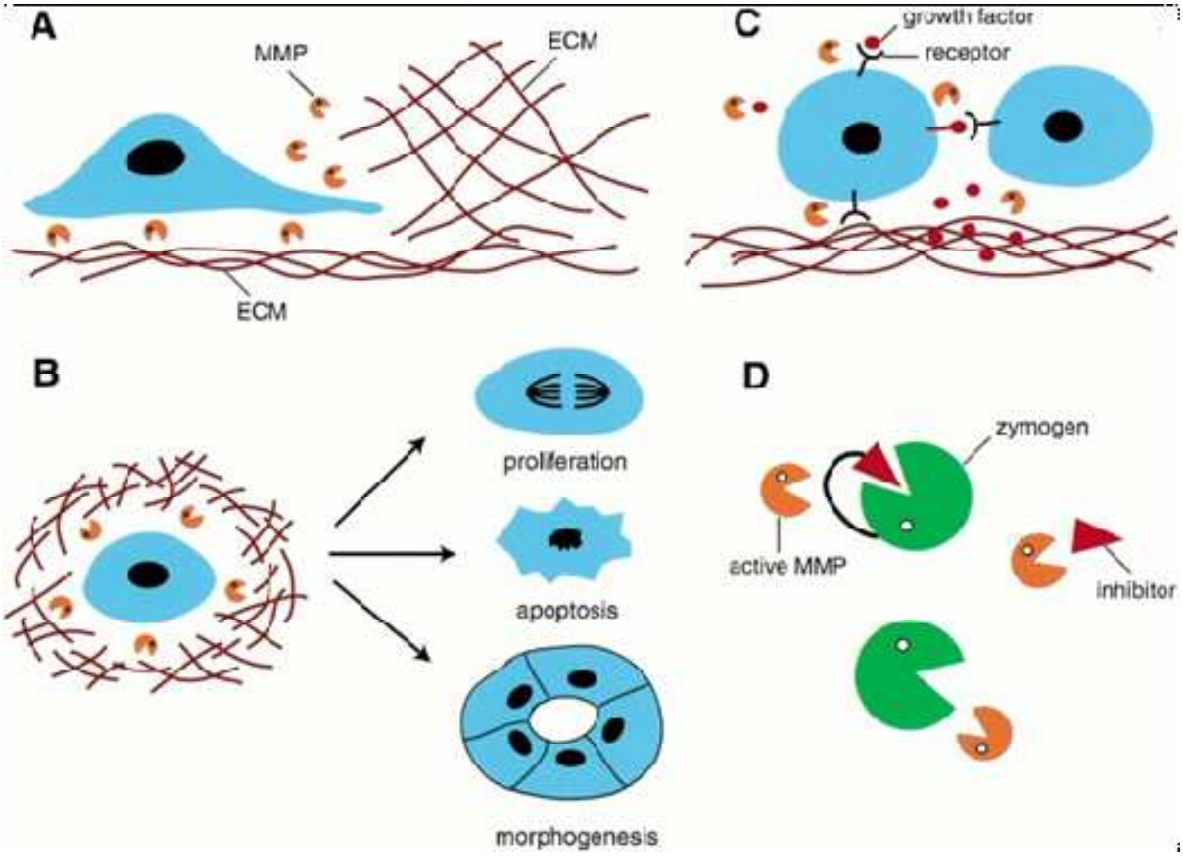

Figure 2.4 A) MMPs may affect cell migration by changing the cells from an adhesive to non adhesive phenotype and by degrading the ECM.

B) MMPs may alter ECM microenvironment leading to cell proliferation, apoptosis, or morphogenesis.

C) MMPs may modulate the activity of biologically active molecules such as growth factors or growth factor receptors by cleaving them or releasing them from the ECM.

D) MMPs may alter the balance of protease activity by cleaving the enzymes or their inhibitors.

2.5. CATALYTIC MECHANISM

There are three catalytic mechanisms which are:-

1) In the first mechanism, Browner M.F. and colleagues proposed the base-catalysis mechanism, carried out by the conserved glutamate residue and the Zn2+ ion.

2) In the second mechanism, the Matthews-mechanism, Kester and Matthews suggested an interaction between a water molecule and the Zn2+ ion during the acid-base catalysis.

3) In the third mechanism, the Manzetti-mechanism, Manzetti Sergio and colleagues [26] provided evidence that a coordination between water and zinc during catalysis was unlikely, and suggested a third mechanism wherein a histidine from the HExxHxxGxxH-motif participates in catalysis by allowing the Zn2+ ion to assume a quasi-penta coordinated state, via its dissociation from it. In this state, the Zn2+ ion is coordinated with the two oxygen atoms from the catalytic glutamic acid, the substrate's carbonyl oxygen atom, and the two histidine residues, and can polarize the glutamic acid's oxygen atom, proximate the scissile bond, and induce it to act as reversible electron donor. This forms an oxyanion transition state. At this stage, a water molecule acts on the dissociated scissile bond and completes the hydrolyzation of the substrate.

2.6. CLASSIFICATION OF METRIX METALLOPROTEINASES

There are 28 types of human matrix metalloproteinases designated as MMP-1,MMP-2,MMP-3,MMP-7,MMP-8,MMP-9,MMP-10,MMP-11,MMP-12,MMP-13,MMP-14,MMP-16, and so on have been recognized, which have been classified as follows (Figure 2.5)

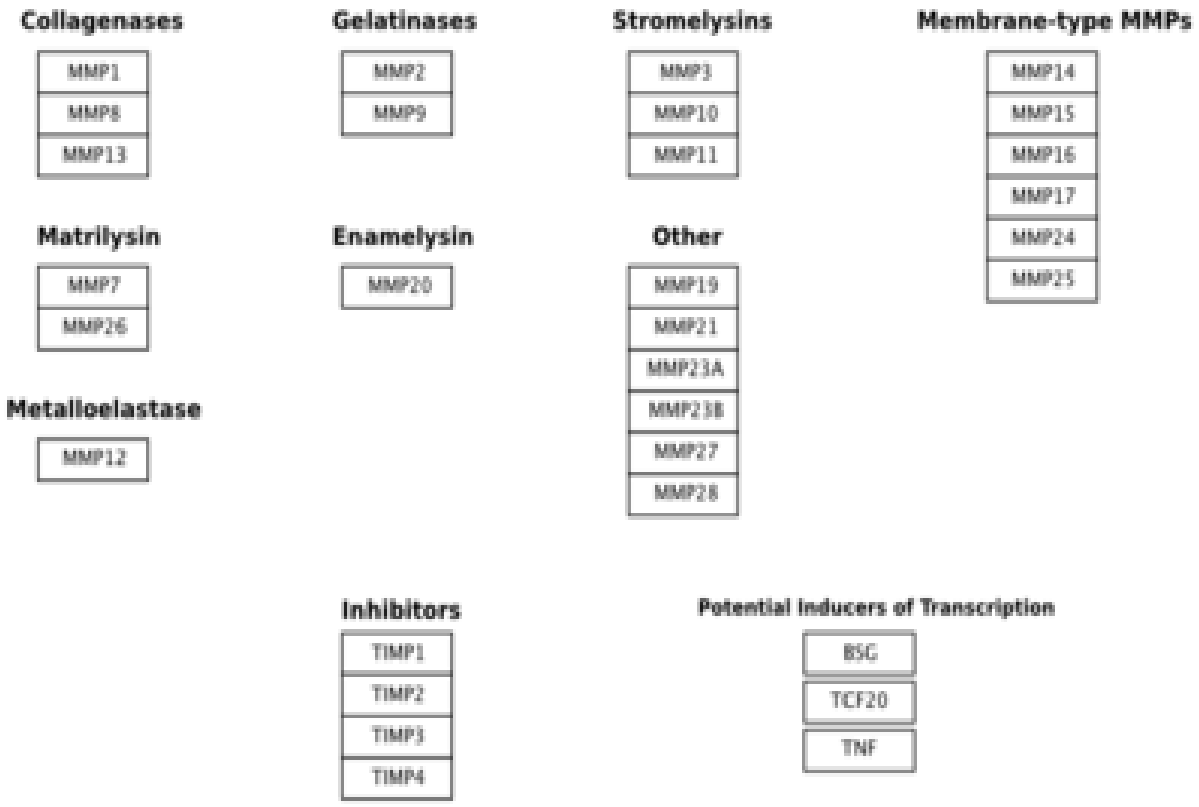

Figure 2.5 : Classification of MMPs under the subfamily of Metrix Metalloproteinases

On the basis of the specificity of MMPs for ECM components, they are divided into collagenases, gelatinases, stromelysins and matrilysins. The common names of the MMPs mirror this classification. Out of eight distinct structural classes of MMPs: five are secreted and three are membrane-type MMPs (MT-MMPs). The MT-MMPs are linked by covalent bonds to the membrane of the cell, the most obvious way of tethering MMP activity to the cell membrane. The other way to localize to the cell surface is by binding to integrins, which is the mode of secreted MMPs or to CD44 or through interactions with cell-surface-associated heparan sulphate

proteoglycans, collagen type IV or the extracellular matrix metalloproteinase inducer (EMMPRIN) [27].

MMP-4, -5 and -6 have been abandoned. When MMP-19 was cloned it was initially called MMP-18. However, an MMP from Xenopus had already received that designation, and therefore this MMP is now known as MMP-19.

The cloning of a partial fragment of human MMP-21 has been described, but the sequence has not been submitted to GenBank and the human enzyme has not been characterized. By similarity with mouse and rat MMP-23.

Gururajan and colleagues identified two new MMP genes, which they called MMP21 and MMP22. The nucleotide sequences of the two genes are almost identical, so they are now designated MMP23A and MMP23B. #Sequence submitted to GenBank (access no. AF195192). GPI, glycosylphosphatidylinositol; MMP, matrix metalloproteinase; MT-MMP, membrane type MMP; PMN, polymorphonuclear neutrophil; PUMP, putative metalloproteinase.

The history and domain knowledge of the MMPs discovery with their history which described in the Figure 2.6. [28]

Name of subclass	Member MMPs	Year of discovery
Minimal domain MMPs	MMP-7/ Matrilysin	1980, 1988
	MMP-26/ Endometase	2001

Simple hemopexin domain containing	MMP-1/ Collagenase-1	1962, 1986
	MMP-8/ Collagenase-2	1968, 1990
	MMP-13/ Collagenase-3	1994
	MMP-18/ Collagenase-4	1996
	MMP-3/ Stromelysin-1	1974,1985
	MMP-10/ Stromelysin-2	1988
	MMP-12/ Metalloelastase	1981, 1992
	MMP-19/ RASI-1	1996
	MMP-20/ Enamelysin	1997
	MMP-22/ CMMP	1998
	MMP-27	2001
Gelatin-binding MMPs	MMP2/ Gelatinase A	1978, 1988
	MMP9/ Gelatinase B	1972, 1989
Furin-activated secreted MMPs	MMP11/ Stromelysin-3	1990
	MMP28/ Epilysin	2001
Transmembrane MMPs	MMP14/ MT1- MMP	1994

	MMP15/ MT2- MMP	1995
GPI-linked MMPs	MMP17/ MT4- MMP	1996
	MMP25/ MT6- MMP	1999
Vitronectin- like insect-less MMPs	MMP21/ XMMP	1998
Cysteine/ Proline-rich IL-1 receptor like domain MMPs	MMP23	1998

Figure 2.6 :Domain knowledge and discovery of MMPs

The different types of MMPs and their name, location, description which are as follows:

MMPs	**Name**	**Location**	**Description**
MMP1	Interstitial collagenase	secreted	Substrates include Col I, II, III, VII, VIII, X, gelatin
MMP2	Gelatinase-A, 72kDa gelatinase	secreted	Substrates include Gelatin, Col I, II, III, IV, Vii, X
MMP3	Stromelysin 1	secreted	Substrates include Col II, IV, IX, X, XI, gelatin

MMP7	Matrilysin, PUMP 1	secreted	membrane associated through binding to cholesterol sulfate in cell membranes, substrates include: fibronectin, laminin, Col IV, gelatin
MMP8	Neutrophil collagenase	secreted	Substrates include Col I, II, III, VII, VIII, X, aggrecan, gelatin
MMP9	Gelatinase-B, 92 kDa gelatinase	secreted	Substrates include Gelatin, Col IV, V
MMP10	Stromelysin 2	secreted	Substrates include Col IV, laminin, fibronectin, elastin
MMP11	Stromelysin 3	secreted	MMP-11 shows more similarity to the MT-MMPs, is convertase-activatable and is secreted therefore usually associated to convertase-activatable MMPs. Substrates include Col IV, fibronectin, laminin, aggrecan
MMP12	Macrophage metalloelastase	secreted	Substrates include elastin, fibronectin, Col IV
MMP13	Collagenase 3	secreted	Substrates include Col I, II, III, IV, IX, X, XIV, gelatin

MMP14	MT1-MMP	membrane-associated	type-I transmembrane MMP; substrates include gelatin, fibronectin, laminin
MMP15	MT2-MMP	membrane-associated	type-I transmembrane MMP; substrates include gelatin, fibronectin, laminin
MMP16	MT3-MMP	membrane-associated	type-I transmembrane MMP; substrates include gelatin, fibronectin, laminin
MMP17	MT4-MMP	membrane-associated	glycosylphosphatidylinositol-attached; substrates include fibrinogen, fibrin
MMP 18	Collagenase 4, xcol4, xenopuscollagenase	-	No known human orthologue
MMP19	RASI-1, occasionally referred to as stromelysin-4	-	

MMP20	Enamelysin	secreted	
MMP21	X-MMP	secreted	
MMP23A	CA-MMP	membrane-associated	type-II transmembrane cysteine array
MMP23B	-	membrane-associated	type-II transmembrane cysteine array
MMP24	MT5-MMP	membrane-associated	type-I transmembrane MMP
MMP25	MT6-MMP	membrane-associated	glycosyl phosphatidylinositol-attached
MMP26	Matrilysin-2, endometase	-	

MMP27	MMP-22, C-MMP	-	
MMP28	Epilysin	secreted	Discovered in 2001 and given its name due to have been discovered in human keratinocytes. Unlike other MMPs this enzyme is constitutivley expressed in many tissues (Highly expressed in testis and at lower levels in lung, heart, brain, colon, intestine, placenta,salivary glands, uterus, skin). A threonine replaces proline in its cysteine switch (PRCGVTD). [29]

The most commonly used groupings (by researchers in MMP biology) are based partly on historical assessment of the substrate specificity of the MMP and partly on the cellular localization of the MMP. These groups are the collagenases, the gelatinases, the stromelysins, and the membrane-type MMPs (MT-MMPs)

2.7. ROLE OF DIFFERENT TYPES OF MATRIX METALLOPROTEINASES

2.7.1. MMP1 (Interstitial collagenase)

Collagen type I, a major component of bone ECM is degraded by Matrix Metalloproteinase 1 (MMP-1). MMP-1 was significantly down-regulated, while TIMP-1 levels were increased, in a time- and pressure-dependent manner in a smooth muscle cell (SMC) mechanical strain model. Fibroblasts, keratinocytes, endothelial cells, monocytes and macrophages express MMP-1. Additionally, a misexpression screen set up to identify molecules required for motoneuron development also resulted in isolation of Mmp1. MMP-1 encoding mRNA was expressed at considerably higher levels in Human OS cells in primary culture than normal human bone cells.

2.7.2. MMP2 (Gelatinase-A, 72 kDa gelatinase)

Whole-mount RNA in in situ hybridization characterized the expression pattern of Mmp2. Expression of Mmp2 widely takes place in the embryonic CNS, which is in contrast to Mmp1. Expression of MMP-2 and beta-catenin loss have a role in the pathogenesis and progression of ESC. Recently, it has been shown that DNAzyme generated against MMP-2 mRNA reduced the expression of the enzymes in vitro, and the size of the C6-glioma in vivo, in the animal model. An important role is played by decreased E-cadherin in the development of both ESC and EEC.

2.7.3. MMP3 (Stromelysin 1)

The pattern of IGFBP-3 degradation products produced by MMP-3 is identical in size to that produced by pregnancy serum. The stromelysin subgroup contains stromelysin-1 (MMP-3). A series of apoE/MMP double knockout mice were used in studies on atherosclerotic plaque stability to indicate that MMP-3 limits plaque growth and enhances plaque stability, and thus plays a protective role.

2.7.4. MMP7 (Matrilysin, PUMP 1)

A big role in the invasion and metastasis of cancer is played by Matrix metalloproteinase-7 (MMP-7), the matrix-degrading enzyme. Studies have shown that oligonucleotides antisense to MMP-7 inhibit the higher rate of spreading of gastric gland cells infected with H. pylori cultures. MMP-7 mRNA was expressed in 53% of primary gastric cancers, but not in normal gastric mucosa, fibroblasts, or mesothelial cells. Induction of MMP-7 takes place during the response of epithelial cell to bacterial infection.

2.7.5. MMP8 (Neutrophil collagenase)

Neutrophil collagenase, a collagen cleaving enzyme, is present in the connective tissue of most mammals. It is also known as (MMP-8) or PMNL collagenase (MNL-CL). It has an exclusive pattern of expression in inflammatory conditions, and is therefore unique among the family of matrix metalloproteinases (MMPs). MMP-8 mRNA and protein were expressed in all the 3 cell types of human atheroma in situ.

2.7.6. MMP9 (Gelatinase -B, 92 kDa gelatinase)

It is also known as 92 kDa type IV collagenase, 92 kDa gelatinase or gelatinase B (GELB) [30]. MMP-9 releases skit and this enables translocation of BM repopulating cells to a permissive vascular niche, which favours differentiation and reconstitution of the stem progenitor cell pool.

2.7.7. MMP10 (Stromelysin 2)

MMP10 gene in humans encodes stromelysin-2 enzyme, which is also known as matrix metalloproteinase-10 (MMP-10) or transin-2.

2.7.8. MMP11 (Stromelysin-3)

In humans, MMP11 gene encodes Stromelysin-3 (SL-3) or (MMP-11) [31,32]. The role of matrix metalloproteinase-11 in neointima formation was tested with the use of a vascular injury model in wild-type (MMP-11+/+) and MMP-11–deficient (MMP-11−/−) mice. Probably, MMP-11 overexpression was associated with the aggressiveness of ovarian carcinoma.

2.7.9. MMP12 (Macrophage metalloelastase)

Investigation into the role of MMP-12 in the development of COPD in human smokers was undertaken in animal models, and it suggested a predominant role for MMP-9 and MMP-12 in the pathogenesis of pulmonary inflammation.

2.7.10. MMP13 (Collagenase 3)

MMP13 gene in humans encodes collagenase 3 enzyme [33]. Expressed by chondrocytes and synovial cells in human OA and RA, MMP-13 is thought to play a critical role in cartilage destruction. It has been reported that in MMP-13 KO mice, degradation of connective tissue growth factor in wound tissue was transiently prevented. MMP-13 remains the major MMP expressed by chondrocytes to degrade their matrix, when they are stimulated with retinoic acid.

2.7.11. MMP14 (MT1-MMP)

Membrane type 1-matrix metalloprotease (MT1-MMP or MMP-14) is a major activator of pro-MMP-2 and is essential for skeletal development. It is generated in vitro by cleavage of membrane-bound native MT1-MMP with several recombinant MMPs, including both active MT1-MMP and MMP-2.

2.7.12. MMP15 (MT2-MMP)

It was found found a correlation exists between the positive nodal status and the expression of 15 mRNA. Hypocellular ECs at E10. 5 were displayed by mice with targeted snai1 knockdown. This was associated with decreased expression of mesenchyme cell markers and down regulation of the matrix metalloproteinase (mmp) family member, mmp15.

2.7.13. MMP16 (MT3-MMP)

According to a numerical nomenclature for matrix metalloproteinases, this is the new name for MT3-MMP [Membrane-type matrix metalloproteinase-3]. In end-stage osteoarthritis, MT3-MMP expression is elevated in human cartilage. PDGF and fibronectin can upregulate MMP-16 expression by cultured vascular smooth muscle cells under pathologic conditions.

2.7.14. MMP17 (MT4-MMP)

MMP-17 (MT4-MMP) is a member of the MT-MMP subfamily. They are anchored to the plasma membrane via a glycosylphosphatidyl inositol (GPI) anchor, which confers these enzymes a unique set of regulatory and functional mechanisms that separates them from the rest of the MMP family.

2.7.15. MMP18 (Collagenase 4, xcol4, Xenopus collagenase)

MMP-18 is expressed in the migrating macrophages, and bands corresponding to mRNA for MMP-18 are present in both HTM and CB tissue.

2.7.16. MMP19 (RASI-1, occasionally referred to as stromelysin-4)

MMP-19 was revealed as a novel mediator in laser capture microscope followed by microarray analysis in hyperplastic epithelial cells adjacent to fibrotic regions. Expressed in human epidermis and endothelial cells, it has roles in cellular proliferation, migration, angiogenesis and adhesion. Yu et al., 2012, identified multiple transcript variants encoding distinct isoforms for this gene [34].

2.7.17. MMP20 (Enamelysin)

An MMP-20 mutation which alters the normal splice pattern and results in premature termination of the encoded protein has been associated with amelogenesis imperfecta.

2.7.18. MMP21 (X-MMP)

MMP-21 enhances tumor invasion and metastasis ability in some solid tumors. MMP-21 expression has been investigated in 296 cases of gastric cancer by immunohistochemistry assay.

2.7.19. MMP23A (CA-MMP)

Unlike other MMPs, MMP23a does not possess the signal sequence. This suggests that it may act intracellularly. MMP-23 has a short prodomain and contains a single cysteine residue that can be part of the cysteine-switch mechanism operating for maintaining enzyme latency.

2.7.20. MMP23B

MMP23B degrades various components of the extracellular matrix. Mmp23b was identified as a gene linked to the genomic locus of an enhancer trap transgenic zebrafish line in which GFP expression was restricted to the developing liver.

2.7.21. MMP24 (MT5-MMP)

TIMPs inhibit all MMPs, except MMP -24.

2.7.22. MMP25 (MT6-MMP)

Membrane-type MMPs (MMP -25, also called MT1-, MT2-, MT3-, MT4-, MT5-, and MT6-MMP, respectively) are structurally similar to the other classes of MMPs but are anchored to the exterior of the cell membrane. It is highly expressed in leukocytes and in some cancer tissues.

2.7.23. MMP26 (Matrilysin-2, endometase)

MMP-26 has 998 mRNA nucleotides and no transcript variant. RT-PCR, immunofluorescence analysis and flow cytometry determined the mRNA and protein expression of MMP-26 by. It is the smallest member of the matrix metalloproteinase. . The encoded protein degrades type IV collagen, fibronectin, fibrinogen, casein, vitronectin, alpha 1-antitrypsin, alpha 2-macroglobulin, and insulin like growth factor-binding protein 1, and activates MMP9 by cleavage.

2.7.24. MMP27 (MMP-22, C-MMP)

mRNAs for MMP-27 are generally expressed at a lower level.

2.7.25. MMP28 (Epilysin)

Matrix metalloproteinase-28 (MMP-28, epilysin) is highly expressed in the skin by keratinocytes, the developing and regenerating nervous system and a number of other normal human tissues. MMP-28 expression is associated with cell proliferation during epithelial repair and is tightly regulated spatially and temporally during wound repair. In primary keratinocytes, expression of MMP-28 is upregulated by treatment with TNF-α [35].

2.8. SUBSTRATE-CLEAVING MECHANISM AND SUBSTRATE-SPECIFITY

MMPs can degrade the majority and minority components of the extracellular matrix. With a few exceptions, namely MMP-11 and MMP-23, most of these enzymes have broad substrate specificity. MMPs not only break down extracellular matrix components, but also act as activators for biologically important molecules. For example, MMP-2, MMP-3 and MMP-7 have the ability to cleave decorin, a protein which interacts with transforming growth factor β-1. Consequently, the growth factor is released into surrounding tissue initiating other processes that may not be directly related to the degradation of extracellular matrix .

2.8.1. Collagenases

This group of MMPs includes MMP-1, MMP-8 and MMP-13. These MMPs degrade α-helices of interstitial collagen (type I, II, III) at two locations, specifically 1/4 and 3/4 fragments, in the place of uncoiled triple-helix (thanks to absence hydroxyproline and higher abundance of hydrophobic groups). Collagenases cleave the α_1 chain of collagen type I in sequence Gly775/Ile776 while the α_2 chain of collagen type I in Gly775/Leu776 regions. Collagenases differ from other MMPs, in that they are only able to cleave collagen helices in the native state at neutral pH by a nondenaturing mechanism. Collagenases significantly differ in their substrate specificity. Neutrophil collagenases (MMP-8) cleave collagen type I substrates, while interstitial collagenases (MMP-1) cleave collagen type III substrates. Although MMP-3 binds to collagen type I, it does not cleave it [36]. MMP-13 cleaves collagen type I and III, but at a slower rate than type II.

In addition to its catalytic properties, De Souza et al. assume that a proline-rich linker in interstitial collagenase mimics collagen conformation, which destabilizes the quaternary structure of collagen triple-helix by forming the proline zipper between linker and substrate. This destabilizing step is critical for further cleavage. After destabilization, the catalytic domain cleaves only one strand of the uncoiled triple-helix in collagen. Afterwards, other MMPs can participate in further cleavage of collagen. Hemopexin-like domains are important in this process, because they can indirectly stabilize pro-collagen-like domains of collagenases. Further studies have shown this domain initially provides non-functional binding sites for substrates over hinge domain to ensure proper spatial arrangement of substrates before their cleavage.

2.8.2. Gelatinases

Gelatinases include MMP-2 and MMP-9 proteins. Gelatinases primarily cleave denatured collagen and intact collagen type IV in basal membranes. They are also able to cleave denatured collagen type V, VII, X, XIV, fibronectin, elastin and aggrecan. MMP-2 is known to cleave native collagen type I. Other studies have shown that MMP-2 binds to intact collagen to prevent autolytic inactivation [37]. In addition to gelatin and other forms of denatured collagen, MMP-9 cleaves a number of other physiological substrates [38,39]. Although sequences cleaved by

gelatinases are still not fully identified, repeated motifs with Pro-XX-Hy- (Ser/Thr), where X is any amino acid residue and Hy is a hydrophobic amino acid residue (Ala, Ile, Leu, Met, Phe, Trp, Tyr and Val) were found to be frequently cleaved. Another group of sequences cleaved by gelatinases include sequence motif Gly-Leu-(Lys/Arg). Substrates containing arginine residues were preferred by MMP-9 [40].

2.8.3. Stromelysins

Stromelysins have relatively broad substrate specificity. The majority of stromelysins cleave non-collagenous extracellular matrix proteins (proteoglycans, glycoproteins, fibronectin and laminin). Collagen type IV is cleaved in globular rather than helical conformation by stromelysins. Moreover, these enzymes can cleave other MMPs. Stromelysin 2 (MMP-10) can degrade the ends of propeptide domains of neutrophil collagenases (MMP-8), cleaving it at the Gly^{78}-Phe^{79} site, leading to the activation of MMP-8 only.

2.8.4. Membrane-Associated MMPs

Membrane-associated MMPs (membrane-type MMPs, MT-MMPs) exhibit similar substrate specificity analogous to free MMPs. They degrade mainly collagen but also other substrates as well. The main difference to other MMPs is their association with the cell membrane. In addition to its location, MT-MMPs also differ in its activity from other types of MMPs. MMP-14 (MT1-MMP) is five to seven times less effective in cleaving hydrolysed collagen type I than its analogue MMP-1. It is however eight times more effective in cleaving gelatin in comparison to MMP-1. MT4-MMP can cleave gelatin and synthetic substrates, but can not cleave collagen type I and IV, fibronectin and laminin. MT2-MMP, MT3-MMP and MT4-MMP can cleave proMMPs prior to activation.

2.8.5. Macrophage Elastase and other MMPs

Macrophage elastase (MMP-12) shares its ability to cleave elastin with other MMPs (gelatinases and matrilysins). It is also able to cleave fibronectin, laminin, collagen, basal membrane, entactin, chondroitin sulphate etc. This enzyme enables macrophages to penetrate basal membrane and, thus, rebuild the inflammatory tissue. MMP-19 also degrades basal membrane. In addition, MMP-20 can degrade tooth enamel, specifically emalogenin, [41]. Many MMPs

(MMP-3, MMP-13, MMP-8 and MT1-MMP) cleave human aggrecan containing globular domain G1 and G2 at Asn^{341}-Phe^{342}.

Diversity of human MMPs are shawn in Figure 2.7.

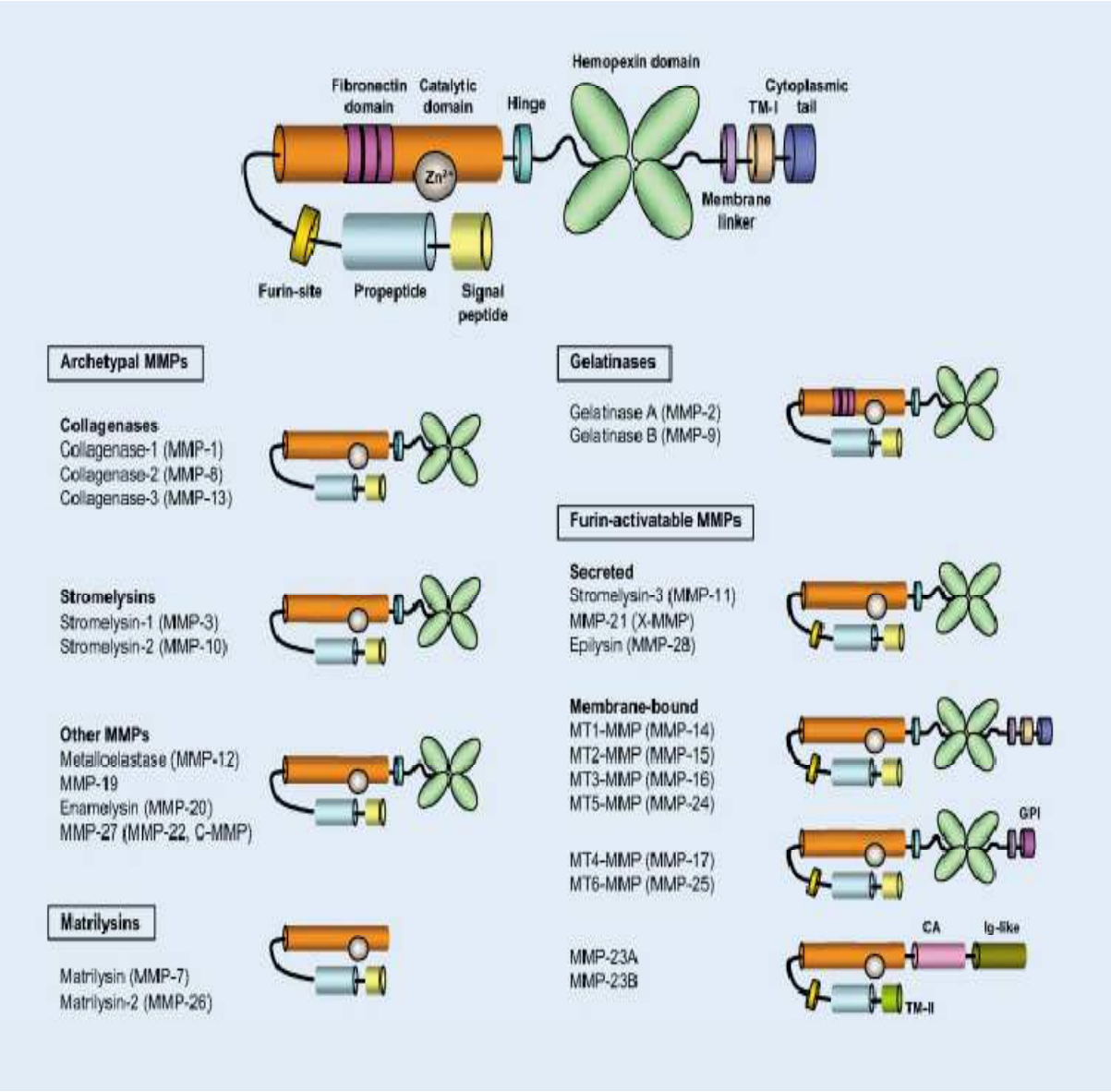

Figure 2.7. Diversity of human MMPs. Structural classification of human MMPs based on their domain organization

2.9. REGULATION OF MMPs

MMPs are synthesized as inactive zymogens. They are kept inactive by an interaction between a cysteine-sulphydryl group in the propeptide domain and the zinc ion bound to the catalytic domain. Their activation requires proteolytic removal of the propeptide prodomain. Activation of majority of MMPs occurs outside the cell by other activated MMPs or serine proteinases. MMP-11, MMP-28 and MT-MMPs can however also be activated by furin-like serine proteinases present intracellularly before they reach the cell surface. Activation of MMP-2 at the cell surface occurs by a unique multistep pathway involving MMP-14 (MT1- MMP) and the tissue inhibitor of metalloproteinases 2 (TIMP-2). During this process, TIMP-2 binds MMP-14 at its amino terminus and pro-MMP-2 at its carboxyl terminus, allowing an adjacent, non-inhibited MMP-14 to cleave the bound pro-MMP-2. MMP-14 does not fully activate MMP-2, as activated MMP-2 is necessary to remove a residual portion of the MMP-2 propeptide [42]. Alternatively, activation of Pro- MMP-2 might also occur by MMP-15 through a mechanism not requiring TIMP-2. An abundant plasmaprotein in tissue fluids, α2-macroglobulin acts as the main inhibitor of MMPs by forming a α2-macroglobulin–MMP complex which binds to a 'scavenger receptor' and gets irreversibly cleared by endocytosis. The debris of the cell is scavenged by scavenger receptors which form a broad class of receptors. They also have other activities, such as adhesion. Similarly, thrombospondin-2 forms a complex with MMP-2, facilitating scavenger-receptor-mediated endocytosis and clearance [43]. Thrombospondin-1 binds to pro-MMP-2 and -9, directly inhibiting their activation [44,45]. TIMPs -1, -2, -3 and -4 remain the best-studied endogenous MMP inhibitors. All of them reversibly inhibit MMPs in a 1:1 stoichiometric fashion [8]. Wang et al., 2000, studied Timp-2-deficient mice and showed that the dominant physiological function of TIMP-2 is activation of MMP-2 [46].

2.10. ACTIVATION OF ProMMPs

2.10.1. Stepwise Activation Mechanism

MMPs can be activated by proteinases or in vitro by chemical agents, such as thiol-modifying agents (4-aminophenylmercuric acetate, $HgCl_2$ and N-ethylmaleimide), oxidized glutathione, SDS, chaotropic agents and reactive oxygens (Figure 2.8). Low pH and heat treatment can also

lead to activation. These agents most likely work through the disturbance of the cysteine-zinc interaction of the cysteine switch. Studies of proMMP-3 activation with a mercurial compound have indicated that the initial cleavage occurs within the propeptide and that this reaction is intramolecular rather than intermolecular. The subsequent removal of the rest of the propeptide is due to intermolecular reaction of the generated intermediates. Recently, studies by Gu et al have shown that NO activates proMMP-9 during cerebral ischemia by reacting with the thiol group of the cysteine switch and forming an S-nitrosylated derivative,[47] a demonstration of the chemical activation of a proMMP in vivo.

Proteolytic activation of MMPs is stepwise in many cases (Figure 8). The initial proteolytic attack occurs at an exposed loop region between the first and the second helices of the propeptide. The cleavage specificity of the bait region is dictated by the sequence found in each MMP. Once a part of the propeptide is removed, this probably destabilizes the rest of the propeptide including the cysteine switch–zinc interaction which allows the intermolecular processing by partially activated MMP intermediates or other active MMPs. Thus, the final step in the activation is conducted by an MMP.

Activation of proMMPs by plasmin is a relevant pathway in vivo. Plasmin is generated from plasminogen by tissue plasminogen activator bound to fibrin and urokinase plasminogen activator bound to a specific cell surface receptor. Both plasminogen and urokinase plasminogen activator are membrane-associated, thereby creating localized proMMP activation and subsequent ECM turnover. Plasmin has been reported to activate proMMP-1, proMMP-3, proMMP-7, proMMP-9, proMMP-10, and proMMP-13.[48] Activated MMPs can participate in processing other MMPs. The stepwise activation system may have evolved to accommodate finer regulatory mechanisms to control destructive enzymes, in as much as TIMPs may interfere with activation by interacting with the intermediate MMP before it is fully activated.

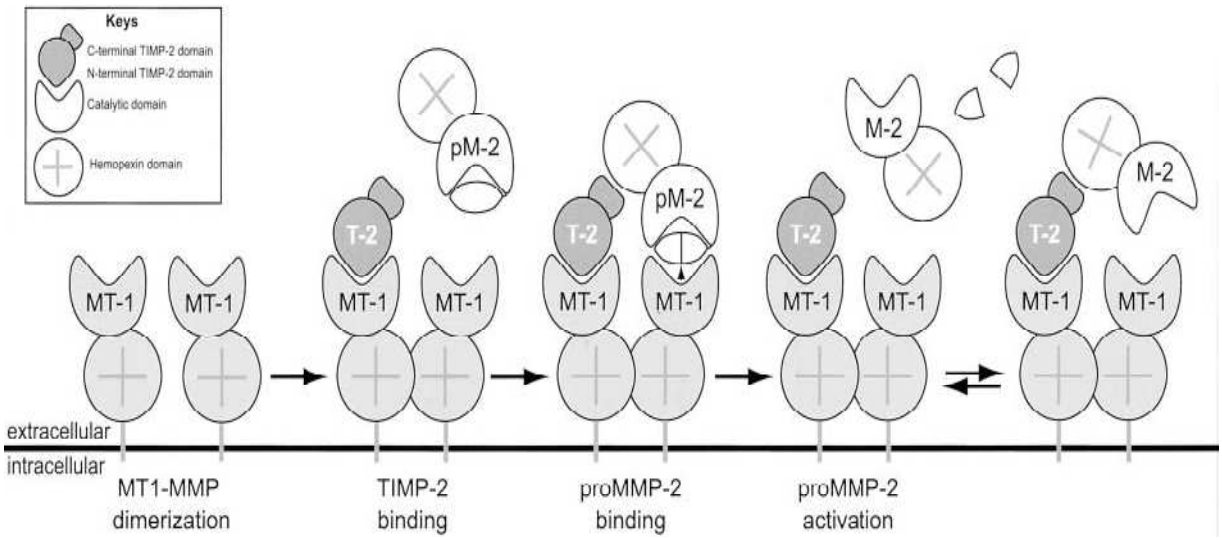

Figure 2.8. Model of proMMP-2 activation by MT1-MMP and TIMP-2. Active MT1-MMP (MT-1) on the membrane binds a molecule of TIMP-2 (T-2), inhibiting its activity. MT1-MMP can form dimers or multimers on the cell surface through interaction of the hemopexin domains. ProMMP-2 (pM-2) subsequently binds to the C-terminal domain of TIMP-2 through its hemopexin domain. The second, active, MT1-MMP then cleaves the bait region of proMMP-2, thereby partly activating it. The MMP-2 (M-2) dissociates from the membrane and is fully activated by intermolecular processing.

2.10.2. Intracellular Activation

Most proMMPs are secreted from cells and activated extracellularly. Pei and Weiss first demonstrated that proMMP-11 (stromelysin 3) is activated intracellularly by furin. ProMMP-11 possesses a furin recognition sequence, KX(R/K)R, at the C-terminal end of the propeptide. Several other MMPs, including the six MT-MMPs,[49] MMP-23, and epilysin (MMP-28),[50] have a similar basic motif in the propeptide. Because these proteins are most likely secreted as active enzymes, their gene expression and inhibition by endogenous inhibitors would be critical for the regulation of activity.

2.10.3. Cell Surface Activation of ProMMP-2

ProMMP-2 is not readily activated by general proteinases. The main activation of proMMP-2 takes place on the cell surface and is mediated by MT-MMPs. This includes MT1- MMP, MT2-MMP, MT3-MMP, MT5-MMP, and MT6-MMP.[51] MT4-MMP does not activate proMMP-2.[52]

MT1-MMP–mediated activation of proMMP-2 has been studied extensively. The unique aspect is that it requires the assistance of TIMP-2. ProMMP-2 forms a tight complex with TIMP-2 through their C-terminal domains, therefore, permitting the N-terminal inhibitory domain of TIMP-2 in the complex to bind to MT1-MMP on the cell surface. The cell surface–bound proMMP-2 is then activated by an MT1-MMP that is free of TIMP-2. Alternatively, MT1-MMP inhibited by TIMP-2 can act as a "receptor" of proMMP-2. This MT1-MMP–TIMP-2–proMMP-2 complex is then presented to an adjacent free MT1-MMP for activation. Clustering of MT1-MMP on the cell surface through interactions of the hemopexin domain facilitates the activation process[53] (Figure 8). Jo et al[54] reported that the maximum enhancement of proMMP-2 activation is observed at a TIMP-2/MT1-MMP ratio of 0.05, suggesting that a large number of free MT1-MMP may surround the ternary complex of proMMP-2– TIMP-2–MT1-MMP for effective proMMP-2 activation.

ProMMP-2 activation by MT2-MMP is direct and independent of TIMP-2.[55] Interestingly, TIMP-4 binds to the proMMP-2 hemopexin domain, and it inhibits MT1-MMP but it does not result in proMMP-2 activation by MT1- MMP.[56] The reason for this is not clear but it may be due to an incorrect molecular assembly with TIMP-4.

MT1-MMP also activates proMMP-13 on the cell surface; this activation is more efficient in the presence of active MMP-2. The activation of proMMP-13 by MT1-MMP is independent of TIMP-2 but requires the C-terminal hemopexin domain of proMMP-13.[57]

2.11. SUBSTRATE SPECIFICITY OF MMPs

Substrate specificities of MMPs have been studied either by identifying the cleavage sites of protein substrates or by a series of synthetic peptide substrates. In general, MMPs cleave a peptide bond before a residue with a hydrophobic side chain such as Leu, Ile, Met, Phe, or Tyr. A peptide bond with a charged residue at this position is rarely cleaved, with the cleavage of the

X-Lys bond by MMP-12 being an exception. The hydrophobic residues fit into the S1 specificity pocket whose size and shape differ considerably among MMPs. In addition to the S1 pocket, other substrate contact sites (subsites) also participate in the substrate specificity of the enzyme.

In some cases, noncatalytic domains influence the enzyme activity, particularly against large extended macromolecules of the ECM. For example, the fibronectin domains of MMP-2 (Figure.9) and MMP-9 are important for its activity on type IV collagen, gelatin and elastin. In collagenase 1 (MMP-1), the loop region just before the catalytic site helix ([183]RWTNNFREY) is essential for collagenolytic activity.[115] Furthermore, the hemopexin domain and the hinge between the catalytic and the hemopexin domains also play key roles in collagenolysis.[58]

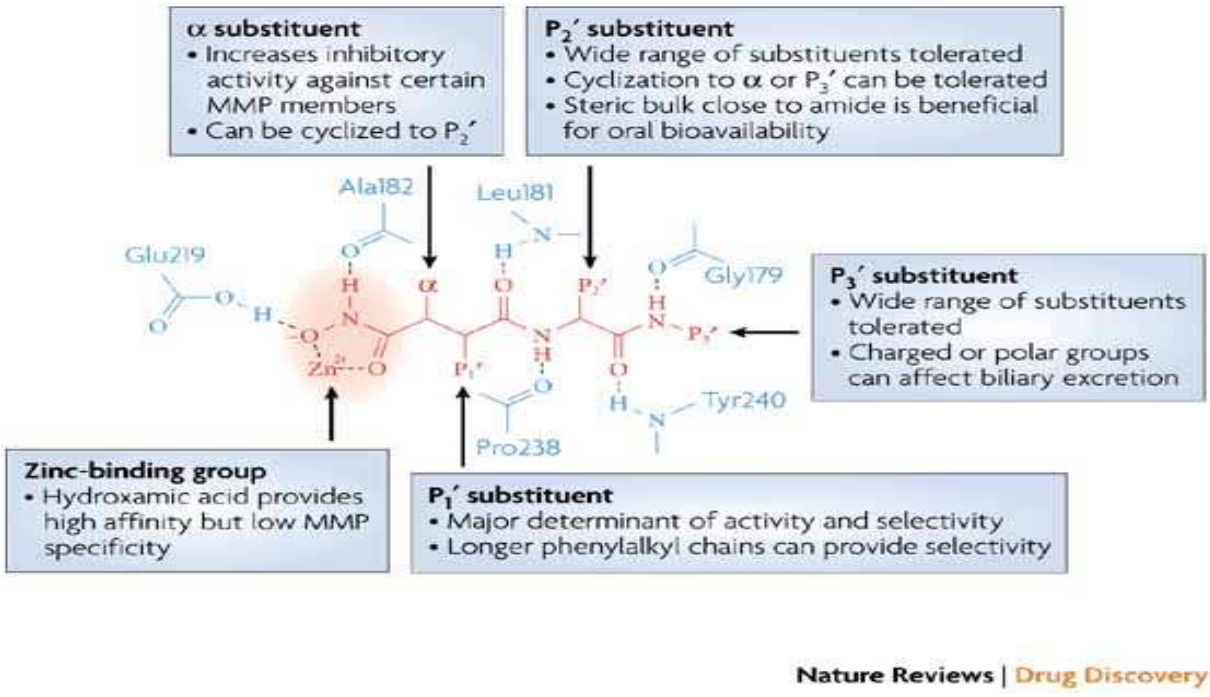

Figure 2.9: Substrate specificity of MMP2

2.12. ROLE OF MMPS IN TUMOR GROWTH, INVASION AND METASTASIS

Tumor growth, invasion, and metastasis are a multistep and complex process that includes cell division and proliferation, proteolytic digestion of the extracellular matrix, cell migration

through basement membranes to reach the circulatory system, and remigration and growth of tumors at the metastatic sites .

The proposed role of MMPs in this process is based on in vitro and in vivo preclinical studies as well as on studies of clinical specimens. MMPs degrade the basement membrane and extracellular matrix, thus facilitating the invasion of malignant cells through connective tissues and blood vessel walls and resulting in the establishment of metastases. In rat embryo fibroblasts, overexpression of MMP-9 degraded the matrix, resulting in enhanced metastatic potential. Tumors in knockout mice lacking specific MMPs exhibit reduced tumorigenesis, angiogenesis and tumor progression. The degradation of the extracellular matrix by MMPs not only facilitates metastasis but also promotes tumor growth by increasing the bioavailability of growth factors that reside in the extracellular matrix and are released during extracellular matrix degradation.

MMP expression, although low or undetectable in most normal tissues, is substantially increased in the majority of malignant tumors. Numerous studies in a variety of tumor types including lung, colon, breast, and pancreatic carcinomas demonstrate overexpression of MMPs in malignant tissues in comparison to adjacent normal tissues. In addition, the plasma and urine levels of MMPs are elevated in patients with cancer compared with healthy subjects . The MMPs in tumor tissues are produced not only by malignant tumors but also by stromal fibroblasts and inflammatory cells. These cells may produce cytokines and proteins that induce the production of MMPs by surrounding cells, creating extracellular networks of MMP secretion and activation. In addition, parallel analyses of tissue samples spanning the process from normal tissue to tumor formation have demonstrated that overexpression of MMPs is a feature of progression to the malignant phenotype. Furthermore, analyses of cellular components derived from primary tumor tissues or their corresponding lymph node metastases demonstrated increased expression of MMPs in the metastatic tissue, indicating that MMP expression is a component of the metastatic process. In addition to the well-documented overproduction and activation of MMPs in malignant tissue, there is now ample clinical evidence that overproduction of these molecules confers a poor prognosis in patients with a variety of malignancies.

Whether specific members of the MMP family are associated with oncogenesis is a matter of debate and varies among the tumor types and stage of lesions studied. Some of this

variability can be attributed to the different experimental conditions and techniques used in different studies. In general, the gelatinases (MMP-2 and MMP-9) have been most consistently detected in malignant tissues and associated with tumor aggressiveness, metastatic potential, and a poor prognosis. However, matrilysin (MMP-7) has been the focus of attention because its preferential expression in early-stage tumors and premalignant lesions may make it a suitable target for chemopreventative strategies.

The role of the MMP family in tumor development is further complicated by the balance of these proteins in relation to TIMPs. The TIMPs have various biologic functions that are, in general, antioncogenic, and the expression of the TIMPs has been associated with less aggressive tumor behavior and favorable prognosis in patients with cancer. For example, the exposure of mouse fibroblasts to TIMP-1 and -2 in vitro inhibited oncogenic transformation by oncogenic viruses whereas the administration of recombinant TIMP-1 to mice injected with B16F10 melanoma cells reduced the number of pulmonary metastases. Furthermore, transgenic mice overexpressing TIMPs displayed resistance to the establishment of intravenously injected malignant cells; conversely, exposure of mouse fibroblasts to TIMP-1 antisense oligonucleotides resulted in the formation of metastatic tumors in nude mice given an injection of malignant cells. Of interest, overexpression of TIMP-3 induces apoptosis in various types of malignant cells, suggesting that TIMPs may play a role in tumor cell death. Thus, the role of TIMPs in vivo is complex, and the expectation that malignant tumors have increased MMP expression accompanied by decreased TIMP expression is probably too simplistic [59,60].

2.13. MMPs INHIBITION AND ANTICANCER ACTIVITY

Synthesis of MMPs is blocked by several agents which prevent them from interacting with the molecules that direct their activities to the cell surface or inhibit their enzymatic activity.

2.13.1. Inhibition of MMP synthesis

MMP synthesis is inhibited directly by transfecting cells with antisense mRNA or oligonucleotides or by targeting mRNA with RIBOZYMES. In mouse models, this means was used to downregulate MMP7 or 9 to reduced tumour burden or metastasis [61,62]. Indirect methods to reduce MMP expression are inhibition of the signal-transduction pathways that induce MMP

transcription. Several drugs in clinical trials inhibit tyrosine kinase receptor signalling and affect MMP expression levels [63]. Halofuginone, a coccidiostat that is used in chickens, is a drug regulating MMP gene expression and experimental cancer-cell metastasis . Coccidiostat is a drug used to treat coccidiosis, an intestinal disease that is caused by a protozoan.

2.13.2. Inhibiting interactions between MMP and other proteins

In order to inhibit interaction of MMP with other proteins, MMP-2 is inhibited from binding to αvβ3 integrin. In clinical practice, this type of strategy could be tested by means of specifically targeting cancer-promoting function and the compound shows promising results in animal experiments .

2.13.3. Exploiting MMP activity

Several cytotoxic agents have been developed that are activated by MMPs. This is useful in treatment of tumours. Cytotoxic agents like recombinant proteins containing anthrax toxin fused to an MMP cleavage site, are activated by MMP cleavage at the cell surface and are internalized by the cell, followed by cell death [64].

2.13.4. Blocking of MMPs

The MMPs are inhibited by specific endogenous (TIMPs), which comprise a family of four protease inhibitors:

• TIMP-1

• TIMP-2

• TIMP-3 and

• TIMP-4

TIMPs might have MMP-independent cancer-promoting activities [65].

Three categories of synthetic MMP inhibitors are:

• The collagen peptidomimetics, which mimic the cleavage sites of MMP substrates. Examples are Batimastat and Marimastat. While Batimastat is no longer tested for the treatment of human cancer, Marimastat has undergone several Phase III clinical trials.

• The collagen non-peptidomimetics, which are synthesized on the basis of the conformation of the MMP active site. Examples include BAY 12-9566, Prinomastat/AG3340, BMS 275291 and CGS 27023A/MMI270. While treatment with BAY 12-9566 in Phase III trials showed poorer survival than for placebo-treated groups, Prinomastat combined with standard chemotherapy did not show beneficial effects compared with chemotherapy alone. Phase II/III clinical trials with BMS 275291 are being recruited [66].

• The tetracycline derivates, which inhibit both the activity and synthesis of MMPs. An example is Col-3 (Metastat) that has entered Phase II trials for Kaposi's sarcoma and advanced brain tumours. A new class of MMP inhibitors are small peptides which can be selected for high specificity for individual MMPs and one such peptide that inhibits MMP-2 and -9 enzymatic activity shows promising effects in animal experiments.

Bisphosphonates, originally developed for the treatment of disturbances in calcium homeostasis and for the prevention of bone metastasis, also inhibit the enzymatic activity of MMPs [67]. Some unconventional MMP inhibitors like AE-941 (Neovastat), an extract from shark cartilage, inhibits MMPs and is now in Phase III clinical trials for the treatment of metastatic non-small-cell lung cancer. A component in green tea being tested in Phase III trial, acts as an MMP-2 and -9 inhibitor in vitro. Acetylsalicylic acid reduces the risk of colon cancer, by directly inhibits MMP-2 activity [68].

2.14. THERAPEUTIC INHIBITION OF MMPs

2.14.1. Strategies for blocking MMP gene transcription

General approaches to inhibit MMP gene transcription target extracellular factors signal transduction pathways or nuclear factors that activate expression of these genes. Targeting MMP transcripts using ribozymes or antisense constructs downregulate MMP production by cancer cells [69].

2.14.2. Extracellular factors

IFN-γ inhibits transcription of several MMPs via the transcription factor STAT1 in diverse human cancer cells [70]. Similarly, IFN-β and IFN-α can also be used for this purpose [71,72]. Blocking of signalling by cytokines or growth factors that upregulate MMPs serves as an alternative approach.

Monoclonal antibodies therapeutically reduce TNF-α-induced MMP production in arthritis and therefore have a potential in cancer too. In a similar strategy for abolishing MMP production in cancer, blocking of IL-1 or epithelial growth factor (EGF) receptors might be useful [73,74]. Retinoids and TGF-β have been reported to downregulate the expression of MMPs and increase TIMP expression, but other studies have reported the opposite [75]. Interestingly, blockade of TGF-β with a soluble TGF-β receptor antagonist inhibits tumour metastasis and the production of active MMP-2 and MMP-9 in mouse models of breast carcinomas [76]. Therefore, proposals to use retinoids or TGF-β as therapeutics to block MMP production in cancer must be reconsidered in light of stimulatory effects of these agents on the production of diverse tumour-associated MMPs. As selecting MMP targets for cancer therapy becomes more complex, the need is to define specific proteases that are involved in a particular tumour at each stage.

2.14.3. Signal transduction

MMP production can also be blocked by targeting the signal-transduction pathways that mediate their induction (Figure 2.10). Halofuginone (an alkaloid from the medicinal plant Dichroa febrifuga) interferes with the TGF-β signalling pathway and inhibits bladder carcinoma metastasis by blocking MMP2 expression [77]. In addition, selective inhibition of p38 MAPK activity with SB203580 abolishes the expression of MMP1, 9 and 13 in transformed keratinocytes and squamous-cell carcinoma cells [78]. Inhibitors of other MAPK pathways, including ERK and JNK, also block the production of some MMPs by tumour cells [79]. Malolactomycin D — a potent inhibitor of transcription that is controlled by the RAS responsive element suppresses the expression of several MMPs and the RAS induced transformed phenotype in NIH3T3 cells, at least in part, by blocking the activation of p38 MAPK [80]. Also, manumycin A — an inhibitor of RAS farnesyltransferase — blocks hyaluronan-mediated MMP-2 secretion in lung carcinoma cells, indicating that RAS signalling is required in this process [81].

Since blocking of general oncogenic signalling pathways-RAS–MAPK, can hamper cancer progression by means of many different mechanisms, therefore it will be crucial to show that therapeutic MMP inhibitors have no deleterious effects on signal transduction.

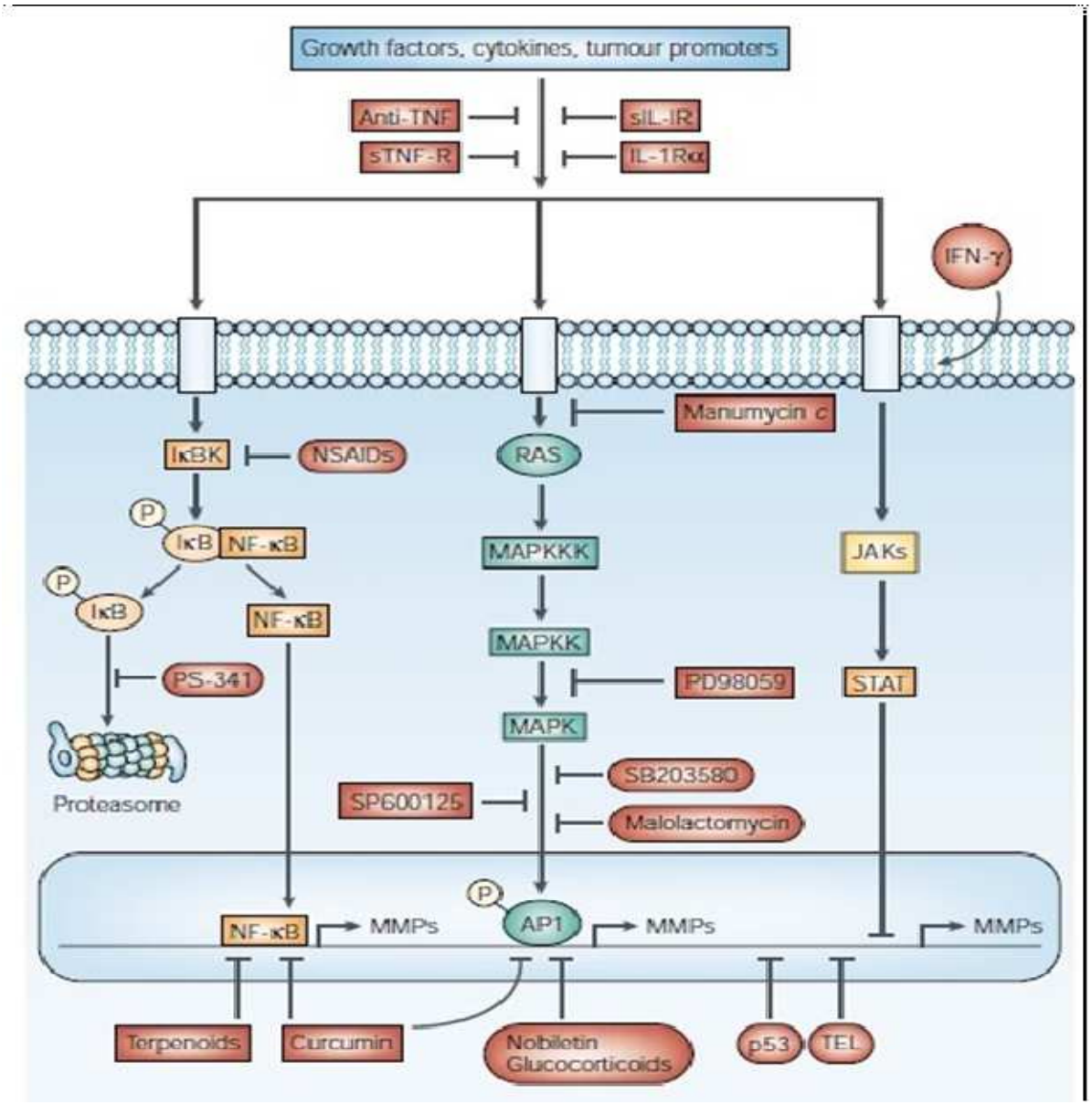

Figure 2.10: Signalling pathways involved in MMP gene transcription, and potential strategies for therapeutic intervention. Compounds that are able to block the transcription of matrix metalloproteinase (MMP) genes at different levels are shown in red boxes. Extracellular factors, such as interferon-γ (IFN-γ) inhibit MMP transcription via the JAK–STAT signalling pathway.

Monoclonal antibodies against tumour necrosis factor-α (anti-TNF), soluble forms of the TNF receptor (sTNF-R), natural antagonists of the interleukin (IL)-1 receptor-α (IL-1Rα) or soluble forms of this receptor (sIL-R) can block signalling pathways initiated by extracellular factors such as TNF-α and IL-1 which induce MMPs in cancer cells. Compounds such as manumycin A, SB203580, malolactomycin, SP600125 or PD98059 act at different levels to block the signal transduction pathways that are associated with MMP transcriptional induction in human tumours. Finally, there are several possibilities to target the nuclear factors that are responsible for MMP transcriptional upregulation. Glucocorticoids, terpenoids, curcumin, nobiletin or NSAIDs (nonsteroidal anti-inflammatory drugs) block the activity of transcription factors such as AP1 and NF-κB which regulate the transcription of several MMP genes. Similarly, restoring the activity of transcription factors such as p53 and TEL which negatively regulate MMP expression and the activity of which is lost in human tumours, could downregulate these genes. IFN-γ, interferon-γ; IκB inhibitor of κB ; IκBK; inhibitor of κB kinase; JAK, JUNactivated kinase; MAPK, mitogen activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen activated protein kinase kinase kinase; NF-κB, nuclear factor of κB; STAT, signal transducer and activator of transcription; TEL, translocation-ETS-leukaemia.

2.14.4. Nuclear factors

A third way to block the upregulation of MMPs in human tumours is by targeting the nuclear factors that regulate these genes (Figure 10). Many different extracellular stimuli and signalling pathways that activate MMP expression converge at the AP1 DNA-binding site. Glucocorticoids interact with the AP1 binding site and prevent the upregulation of MMPs [82] but this treatment can affect the expression of many genes and have several side effects. Natural products such as nobiletin — a flavonoid obtained from Citrus depressa — have been shown to inhibit AP1 binding activity and suppress both the production of MMP-1 and MMP-9 by human fibrosarcoma cells and the invasive properties of these cells [83]. Similarly, curcuminoids, natural products of the Indian spice turmeric, inhibit MMP-9 expression by interfering with AP1-induced transcription [84].

Another factor that can be targeted to prevent MMP transcription is NF-κB in cancer [85]. Interestingly, PS-341 — a proteasome inhibitor that blocks the degradation of inhibitor of κB

(IκB) and thereby maintains NFκB in an inactive status, might be effective in treating multiple myeloma and other cancer types in humans. Synthetic triterpenoids and non-steroidal anti-inflammatory drugs also interfere with the NF-κB pathway [86,87].

Some transcription factors such as p53, PTEN (phosphatase and tensin homologue) and ETS transcription factor (TEL) are involved in negative regulation of MMP expression. Their activity is commonly lost during tumour progression which leads to an increase in the proteolytic capacity of tumour cells [88]. Adenoviral delivery of wild-type p53 into squamous-cell carcinoma cells that carry mutant forms of p53 inhibits expression of MMPs and invasive properties, independently of the pro-apoptotic effect of p53 on these cells [89].

2.14.5. Strategies for Blocking proMMP Activation

Inhibitors of plasmin can prevent cleavage of proMMP and combined with administration of MMPIs can profoundly reduce tissue highlighting the potential for similar combinatorial treatment of cancer [90]. MT-MMP, an MMP-activating enzyme, is also overexpressed by different malignant tumours. Together with their general proteolytic behaviour, the MT-MMPs should be considered primary targets.

Anti-inflammatory cytokines, such as IL-4 and IL-13, have been shown to interfere with the proMMP activation process rather than with enzyme expression [209]. Natural products such as green tea catechins, have also been reported to block the MT1-MMP-dependent activation of proMMPs [91]. Similarly, since MMP-3 is a well-characterized activator of proMMPs, inhibitors of this enzyme will also prevent the activation of other proMMPs.

2.14.6. Proprotein convertase inhibitors

A selective furin inhibitor, α1-PDX, has been shown to prevent tumour growth and invasion of human cancer cells [92,93]. Activation of MT1-MMP was prevented by Furin inhibitions, resulting in reduced processing of proMMP-2.Similar results were obtained using a synthetic furin inhibitor. However, the effect of these inhibitors on the activation of the secreted convertase sensitive MMPs, such as MMP-11 (stromelysin-3), which is strongly expressed by tumour stroma has not been reported. Convertase inhibitors block MMPs but in view of the essential

roles of convertases in protein processing in many tissues, side effects could limit the dosage that can be administered and therefore limit efficacy in humans.(Figure 2.11)

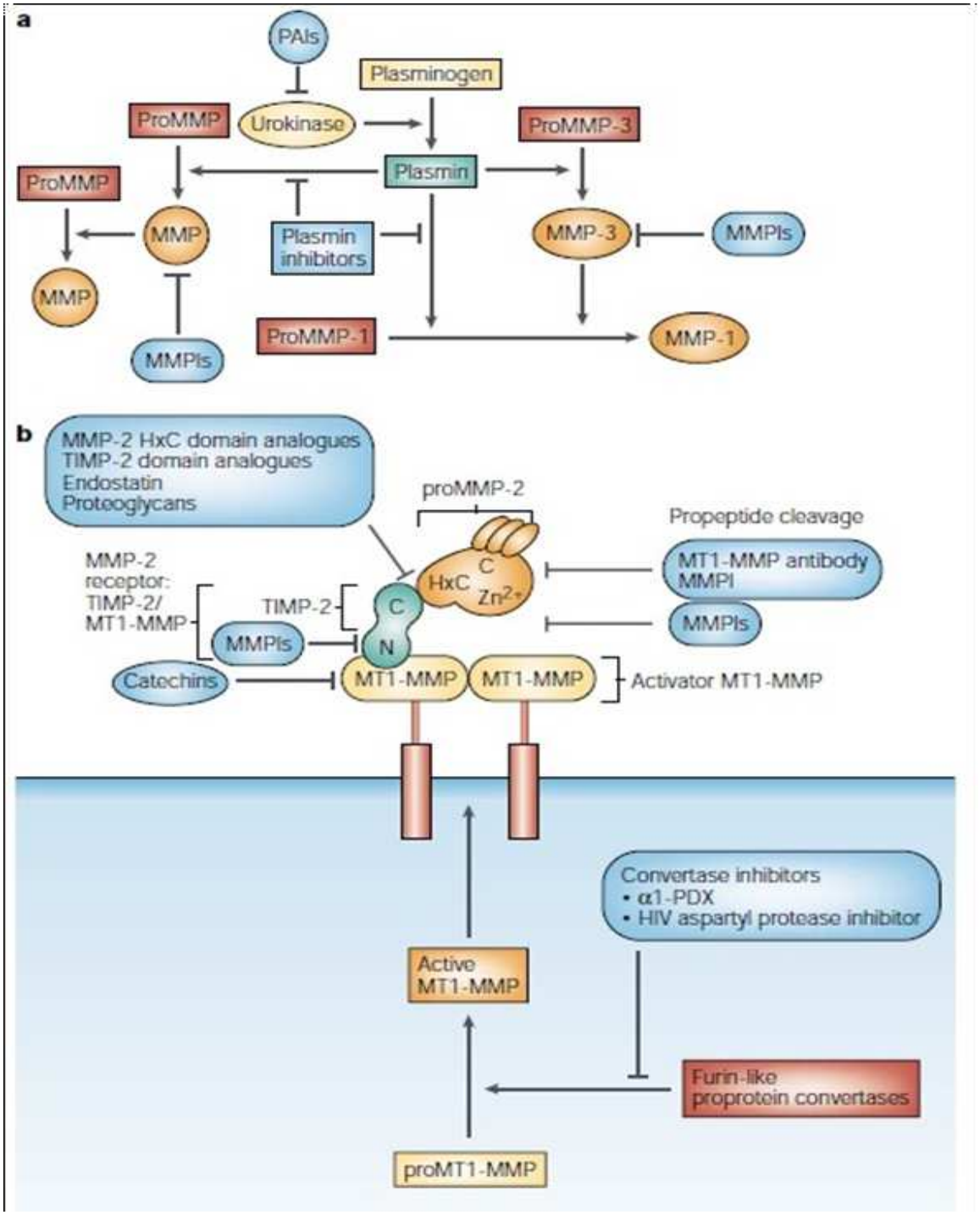

Figure 2.11**:** Strategies for blocking proMMP activation.

a) Active MMPs are generated through a multistep proteolytic cascade that involves plasminogen cleavage by urokinase to create plasmin, which then cleaves proMMPs to create active MMPs. Some of these MMPs go on to cleave other proMMPs. MMP inhibitors (MMPIs) block MMP generation from proMMPs. Serine proteinase inhibitors (PAIs) of urokinase block plasmin generation and plasmin inhibitors block the proMMP conversion to MMPs.

b) Blockade of cell-surface and furin-mediated activation of MMPs. ProMMP-2 activation occurs after the formation of a ternary complex that contains proMMP-2 linked to cell-surface MT1-MMP via a tissue inhibitors of metalloproteinase (TIMP)-2 bridge. The TIMP-2 inhibitory amino (N) domain binds to the active site of MT1-MMP, inhibiting its proteolytic activity. TIMP-2 also binds MMP- 2 outside the catalytic domain (C) on the outer rim of the hemopexin carboxy-terminal domain (HxC), at the junction of hemopexin modules III and IV . The crystal structure of the complex proMMP-2/TIMP-2 has shown that the inhibitory N domain of TIMP-2 is not in contact with the HxC domain and is also distant from the catalytic site of MMP-2 [94]. MT1-MMP has been shown to dimerize in forming the trimolecular complex on the cell surface with MMP-2. MT2-MMP does not require TIMP-2 to bind or activate MMP-2 [56] and it is not known whether the other MT-MMPs form a complex with TIMP-2. Compounds that interfere with the activation of proMMP-2 include catechins, anti-MT1-MMP antibody and MMPIs. MMP-2 HxC domain analogues and TIMP-2 domain analogues disrupt the TIMP-2/MMP2 interaction and prevent MMP-2 activation. Endostatin and proteoglycans which form complexes with MMP-2 inhibit processing and activation by MT1-MMP. Active MT1-MMP is generated through a proteolytic cascade from proMT1-MMP which are generated by furin-like proprotein convertases. These convertases can be inhibited by inhibitors such as α1-PDX and human immunodeficiency virus (HIV) aspartyl protease inhibitors.

2.14.7. MMPs Inhibitors :

MMP activation can also be blocked by the use of thrombospondin- 1 — an anti-angiogenic factor that inhibits proMMP-2 and proMMP-9 activation [44,45]. Similarly, thrombospondin-2 promotes MMP-2 endocytosis via the low-density lipoprotein-receptor related protein pathway [43]. Endostatin forms a complex with MMP-2 and inhibits processing and activation by MT1-MMP [95] which might partially explain its antiangiogenic activity. Similarly proteoglycans, such

as testican-3 and its splice-variant gene product N-Tes, can suppress proMMP-2 activation that is mediated by MT-MMPs, with the subsequent abrogation of invasive properties of glioma cells [96]. A potent inhibitor of proMMP-2 activation in vitro is recombinant hemopexin, indicating the feasibility of targeting this interaction to block MMP-2 activation in vivo [55,97]. Analogues of the TIMP-2 C-terminal domain might be used to compete for TIMP-2 binding to the MMP-2 hemopexin C-terminal domain, and so might prevent trimolecular complex formation that is required for MMP-2 activation. Accordingly, MMPIs have both a direct effect in inhibiting active site MMPs and an indirect effect in blocking TIMP-2 binding and MMP-2 activation by MT1-MMP. Important matrix metalloproteinases inhibitors are shown in Figure 2.12.

Inhibitor	Structure	Specificity	Comments
Marimastat (BB-2516)	Peptido mimetic	Broad spectrum	Survival benefit in a subset of gastric cancer patients Survival benefit in glioblastoma multiforme patients in combination with temozolomide Survival rate similar to gemcitabine in pancreatic cancer No survival benefit in SCL, NSCL and ovarian cancer patients
Tanomastat (BAY 12-9566)	Non-peptido mimetic	MMP-2, 3, 9	Development halted because treated patients showed poorer survival than controls
Prinomastat (AG3340)	Non-peptido mimetic	Broad spectrum	No survival benefits in NSCL cancer patients No difference in progression of prostate carcinomas.
Metastat (COL-3)	Tetracycline derivative	Gelatinases	Multiple mechanisms of action against MMPs Currently recruiting Kaposi's sarcoma patients
Neovastat (AE-941)	Shark cartilage extract	Broad spectrum	Multiple mechanisms of action on MMPs Currently recruiting renal-cell carcinoma, multiple myeloma and NSCL cancer patients
BMS-275291	Non-peptido mimetic	Broad spectrum	Currently recruiting NSCL cancer patients
MMI270	Non-peptido mimetic	Broad spectrum	Anti-angiogenic and anti-metastatic effects in animal models Phase I studies in patients with advanced malignancies

Figure 2.12, Matrix metalloproteinase inhibitors in clinical development for cancer therapy.

MMPs, matrix metalloproteinases; NSCL cancer, non-small-cell lung cancer; SCL cancer, small-cell lung cancer. Table adapted from Coussens et al., 2002; Hidalgo et al., 2001 [98,99].

2.14.8. Natural Compounds as MMP-Inhibitors

Matrix metalloproteinases have been heralded as promising targets for cancer therapy on the basis of their massive up-regulation in malignant tissues and their unique ability to degrade all components of the extracellular matrix. Preclinical studies testing the efficacy of MMP

suppression in tumor models were so compelling that synthetic metalloproteinase inhibitors (MPIs) were rapidly developed and routed into human clinical trials. The results of these trials were, however, disappointing.

2.14.9. Natural inhibitors of MMPs

Natural inhibitors of MMPs- TIMPs, were also used to block MMPs activity. Although they have demonstrated efficacy in experimental models, TIMPs may exert MMP-independent promoting effects [100]. To avoid the negative results and toxicity issues raised by the use of synthetic MMPIs, one answer was provided from the field of natural compounds. One compound taken into consideration was extracted from shark cartilage. Oral administration of a standardized extract, neovastat, exerts anti-angiogenic and anti-metastatic activities and these effects depend not only on the inhibition of MMPs enzymatic activity but also on the inhibition of VEGF. Another natural agent that has anticancer effects is genistein, a soy isoflavonoid, structurally similar to estradiol. Apart from its estrogening and anti-estrogenic properties, genistein confers tumor inhibition growth and invasion effects, interfering with the expression ratio and activity of several MMPs and TIMPs [101].

In 2004, Lambert et. al. reported that the matrix metalloproteinase inhibitors (MMPIs) may be derived from natural resources such as herbs, plants, fruits and other agriculture products [102]. New and potentially beneficial compounds isolated from these sources were shown to exhibit some degree of MMPI activities but they were far less potent and specific than the TIMP family. These natural compounds included long chain fatty acids, epigallocatechin gallate (EGCG) and other polyphenols and flavonoids.

Perhaps the most thoroughly studied class of natural MMP inhibitors are the endogenous tissue inhibitors of metalloproteinases (TIMPs) of which four are currently known, designated as TIMP-1 through -4. It is assumed that the natural ratio of MMPs to TIMPs is tightly regulated and a disruption in the natural balance between these two families is often associated with the progression of multiple disease states. Each of the four TIMPs forms a complex with the MMPs in a 1:1 stoichiometry, exhibiting high affinity but varying degrees of selectivity.

2.15. MMPIs FROM MARINE NATURAL PRODUCTS

2.15.1. Marine saccharoid MMPIs: Most of the marine saccharoid MMPIs inhibit MMP by direct down regulation of MMP-9 transcription or via inhibition of activator protein-1(AP-1) pathway or nuclear factor κB (NF-κB) pathway.

Marine saccharoid MMPIs exhibit high MMPs inhibitory activity either by direct inhibition of the enzyme or by inhibiting the expression of MMPs. These have also shown low toxicity levels. However, due to high molecular weight of theses MMPIs, the structureactivity relationship and the mechanism of the activity is hard to be addressed. If these shortcomings are overcome in the future, marine saccharoid MMPIs have a great potential to be used in clinical applications.

2.15.2. Marine flavonoids and polyphenols MMPIs: Flavonoid glycosides, isorhamnetin 3-O-b-D-glucosides, and quercetin 3-O-b-D-glucoside were isolated from Salicornia herbacea and their inhibitory effects on matrix metalloproteinase-9 and -2 were evaluated in human fibrosarcoma cell line [103].

Flavonoids and polyphenols MMPIs have excellent MMPs inhibitory activities; however, they show a high toxicity level. Therefore, the pharmaceutical applications of these MMPIs are limited. Researchers should pay attention to reduce their toxicity levels by altering the structure in a way that it preserves the bioactivity. Then this class of MMPIs will gain a huge potential to be used in clinical applications.

2.15.3. Marine fatty acid MMPIs: Long-chain fatty acids can inhibit MMPs. However for different MMPs, the degree of inhibition is different. Oleic acid and elaidic acid can inhibit MMP-2 and MMP-9 with the micromole Ki values, although their inhibitory effects on collagenase-1 (MMP-1) are weak [104]. The fatty acid chain length and its degree of saturation is related to the level of inhibition, as the fatty acids with long carbon chains show stronger inhibition than the short ones and the non-saturation degree shows a positive correlation to the overall inhibitory capacity of the fatty acid chains. Fatty acids bind to neutrophil elastase while the parinaric acids are inhibitors of neutrophil elastase. The fatty acids bind to plasmin for

example, oleic acid can modulate fibrinolysis. It is well known that the marine fishes are rich in omega-3 long-chain polyunsaturated fatty acids (ω_3 LC-PUFAs), especially eicosapentaenoic (EPA) and docosahexaenoic acid (DHA). Suzuki et al. found that the inhibition of lung metastasis of a colon cancer cell line by EPA and DHA was associated with a reduced activity of MMP-9. A wide range of biological activities such as cytotoxicity, antimicrobial, antifouling and enzyme inhibition are shown by acetylenic fatty acids isolated from marine sponges. Sodium 1-(12-hydroxy) octadecanyl sulfate inhibits MMP-2. Callysponginol sulfate A, extracted from the marine sponge, Callyspongia truncate, inhibits recombinant MT1-MMP.

2.15.4. Other marine natural products MMPIs: The compounds extracted from shark cartilage (such as Neovastat, AE-941, U-995) have been investigated for their potential use as MMPIs. Neovastat inhibits enzymatic activity of MMP-2 with minor inhibition of MMP-1, -7, -9 and -13. TIMP-like proteins within AE-941 could be responsible for its specific MMP inhibitory property [105]. The Atlantic cod (Gadus morhua) muscle contains a 21-kDa proteinase inhibitor which has properties similar to human TIMP-2. The inhibitor was found to inhibit the gelatin-degrading enzymes present in the gelatin-bound fraction. In addition, it inhibited gelatinolytic activity obtained from a human macrophage cell medium rich in MMP-9 [106].

2.15.5. Marine plants: Metabolites from marine plants have outstanding biological activities. A highly effective anticoagulant and antiproliferative agent, sulfated polysaccharide, from brown alga Ecklonia cava, exhibited a promising antiproliferative effect on human promyelocytic leukemia (HL-60) and human leukemic monocyte lymphoma (U-937) cells. Fucoidan extracts from sea weed Cladosiphon novae reduce the cellular invasiveness in human fibrosarcoma HT1080 cells by suppressing the activity of MMP-2 and MMP-9. Further, it has been reported that these fucoidan extracts suppresse the expression and secretion of an angiogenesis factor, vascular endothelial growth factor (VEGF); thereby reporting the inhibitory effects on invasion and angiogenesis of tumor cells [107].

Extracts from Eisenia bicyclis, Ecklonia cava and Ecklonia stolonifera have strongly reduced MMP-1 expression via inhibiting both NF-kappa B and AP-1 reporter. Free radical scavenging activity of phlorotannins from Ecklonia species has been reported. Dieckol from marine brown alga, E. cava has been reported to suppress LPS-induced production of nitric

oxide (NO), prostaglandin E2 (PGE2), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in murine BV2 microglia; thus establishing dieckol as a potent anti-inflammatory and neuroprotective agent. Flavonoid glycosides isolated from this plant have inhibited the expression of MMP-2 and MMP-9 and elevated the TIMP-1 expression in human fibrosarcoma (HT1080) cells. Moreover, the down regulation of MMP-9 and MMP-2 by these flavonoids is due to the interference with the transcription factor AP-1, there by suggesting that these flavonoid glycosides can be used as potent natural chemopreventive agents for cancer. Phlorotannins from brown alga E. cava have been reported to have inhibitory activity on MMP-2 and MMP-9, signifying the role of phlorotannins as potential and safe marine derived MMPIs.

2.16. MISCELLANEOUS NATURAL PRODUCTS

Screening has led to the discovery of both synthetic and natural MMP inhibitors. The latter include tetracyclines, such as doxycycline and minocycline, for which it has been found that chemical modification can separate MMP activity from antibiotic activity. Actinonin has been identified as an MMP inhibitor and it is a succinyl hydroxamic acid that bears close structural similarity to compounds obtained by substrate based design. Although significant advances have been made in inhibitor design, it is still not clear how to design compounds that specifically inhibit individual MMPs inspite of the available structural data. This remains a major challenge for MMP inhibitor medicinal chemistry.

2.17. OTHER NATURAL COMPOUNDS

Fujita et al. in 2003 reported that ageladine A, a fluorescent alkaloid isolated from the marine sponge Agelas nakamurai inhibits MMP-1, MMP-8, MMP-9, MMP-12, and MMP-13.This compound could also inhibit MMP-2 but N-methylated derivatives did not inhibit MMP-2. The inhibition is not due to Zn2+ chelation, as ageladine is not capable of chelating to Zn^{2+}, and a kinetic analysis indicated that the inhibition was not competitive. In addition, bovine aortic endothelial cell migration and vascular formation by murine ES cells were significantly inhibited by this compound [108].

2.18. FUTURE PERSPECTIVE

Research into neoepitopes will provide important and novel means of diagnosis, prognosis and increasing treatment efficacy in cancer. However, to fully take advantage of neoepitopes as highly valuable cancer biomarkers, it is very important to understand the physiological mechanisms and signalling pathways that regulate their generation. Thus, the ultimate goal of new diagnostic tests should be to use highly reliable non-invasive mechanism-based biomarkers. At present, receptors, cell adhesion molecules, growth factors and enzymes, with their related protein substrates (e.g., MMPs and extracellular matrix components), are all hot research areas in the development of cancer drugs and diagnostic assays [109].

Further evidence establishing the usefulness of β interferons and glatiramer in the treatment of relapsing-remitting multiple sclerosis has been advanced. Interferon-β-1b was also shown to be efficacious in secondary progressive multiple sclerosis. There are more than 20 MMPs identified that share several common features: signal peptides, prodomain, and prodomain, and catalytic domain, with at least eight of these proteins clustered on chromosome 11 (MMPs-1, MMPs-3, MMPs-7, MMPs-8, MMPs-10, MMPs-12, MMPs-13, and MMPs-20), probably due to a gene duplication event [110]. Johnson et al., reported in 2001, that although the healthy adult lung is not a major source of MMPs, parenchymal cells such as airway epithelium, fibroblast, and smooth muscle have the capacity to express active MMPs following stimulation by a variety of agents such as infectious pathogens, environmental toxins, growth factors, and cytokines [111]. Lopez-Boado et al. 2000 reported a 25-fold induction of MMP-7 in the lung epithelial cells following infection with Escherichia coli and Pseudomonas aeruginosa, which could explain the up regulation of this enzyme in the airway of cystic fibrosis patients who are commonly infected with these bacteria. It also has been shown that proinflammatory cytokines such as interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), upregulate the expression of MMP-9 in human airway epithelial cells following a 1-day treatment. Additionally, inflammatory cells invading the lung during the course of COPD are also a major source of different MMPs. It has been shown that the neutrophils and macrophages, the predominant inflammatory cells in the lungs of COPD patients, have the capacity to release MMPs -2, MMPs -3, MMPs -7, MMPs -9, and MMPs -12.

2.19. FUTURE OF CELL UTILIZATION FOR DISC DISEASE

Despite the growing number of research data on cell-based experimental therapy for intervertebral disc (IVD) disease, it is clear that we do not know much about native disc cells and their microenvironment. This lack of information is a major obstacle in building a strategy for the treatment of IVD disease. Investigating the specific differentiation status of native IVD cells and their homeostasis will surely provide more ideas and clues for efficient therapeutic approaches. Although cell-based therapy for IVD disease is still in its infancy, the stage of testing a variety of cells for injection should be toned. To progress to the next step, we should be investigating what exactly IVD cells are, and how they control their homeostasis, along with various studies optimising parameters, such as cell dosage and culture period and the severity of IVD degeneration in the recipient [112].

Moreover, there is the need for attention to the stage and type of cancer that is likely to be evaluated in clinical versus preclinical studies. For example, the selection of advanced pancreatic and lung cancers for clinical trials was based on considerations such as expected survival time and patient availability, both of which affect the time and financial resources required to achieve statistically significant results. Patent issues, competition and impatience contributed to the decision to proceed at an unprecedented pace in an inappropriate setting and these factors will undoubtedly continue to influence drug development decisions in the future.

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3. GLUTAMINE ANALOGS AS POSSIBLE ANTICANCER AGENTS:

3.1. Rationale of choosing the glutamine analogs:

The development of cancer therapy is the urgent aim for science today. Growing knowledge about the metabolism of cancer cells provides new interesting hints concerning the metabolic targeting of the treatment and searching for new drugs inhibiting the growth of cancer[1].

The Biochemical aspects of cancer metabolism and possible use of this for targeting the therapy into mainstream metabolic enzymes is gaining the momentum. Main emphasis is placed on the possible effect of glutamine analogs as a nutrient supporting both the cancer growth and to fight against the cancer.

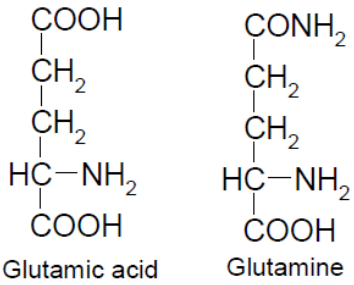
In cancer, mostly there is depletion of glutamine, as cancer cells consume glutamine at large scale that is why there is lack of glutamine in the skeletal muscle[2]. Glutamine plays very important roles in tumor cell.[3]

Previously, in our laboratory successfully developed new antitumor or anticancer chemical entities, including glutamine and glutamamide analogs that possibly act as glutamine antagonists.[4-6] So In the present work, it was tried to develop some isoglutamine analogs which are substituted with different groups. These isoglutamine analogs may be a more active anticancer agents than the earlier series of compounds.

3.2. Introduction:

Glutamine is an amino acid (a building block for proteins) that is used in the biosynthesis of proteins, found naturally in the body.[7] The amino acid glutamine and glutamic acid are closely related in chemical sense are important amino acid. L-glutamic acid is considered a nonessential amino acid, which means the body is able to produce it on its own and one does not need to get it from the food While the body can make L-glutamine, it is considered a conditionally essential amino acid. That means it's essential in the diet under certain conditions including times of major illness such as trauma, surgery, bone marrow transplant or intense chemotherapy. During these times one may need to supplement with L-glutamine.[8]

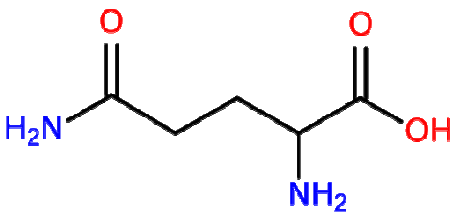
The human body is able to produce L-glutamine itself is , from L-glutamic acid through the glutamate ammonium ligase. Considering the numerous metabolic processes glutamine is a part of, it is not surprising that it is the amino acid with the highest concentration in blood plasma, musculature and cerebral and spinal fluid. At 60%, it represents the largest amount of free amino acids in the body.[9]

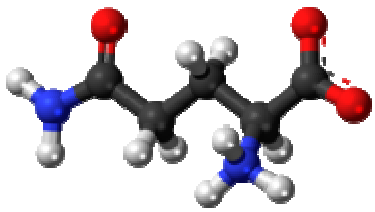


Glutamic acid is one of the 20-23 proteinogenic amino acids and its codons are GAA and GAG. The carboxylate anions and salts of glutamic acid are known as glutamates.

Glutamine contains an α-amino group (which is in the protonated −NH3+ form under biological conditions) an α-carboxylic acid group (which is in the deprotonated −COO− form under biological conditions), and a side chain amide which replaces the side chain hydroxyl of glutamic acid with an amine functional group, classifying it as a charge neutral, polar (at physiological pH) amino acid.[10]

3.3.Properties:[11]





Structure of L-Glutamine

Molecular formula:	$C_5H_{10}N_2O_3$
Molar mass:	146.14 g mol^{-1}
Melting point:	decomposes around 185°C
Solubility in water:	soluble
Chiral rotation [α]D:	+6.5° (H_2O, c = 2)
Bulk density:	640 kg/m^3
pH value:	4.0 - 6.0 (25 g / l, H_2O, 20 ° C)

3.4.Metabolism of Glutamine:

3.4.1. *Metabolism of Glutamine in Healthy Cells*:

Glutamine is one of 20 amino acids, commonly existing in every protein. The central point in the metabolism of majority of amino acids is glutamine.[12]

According to the body's metabolic needs, hepatocytes serve as a glutamine producer and consumer. Mainly glutamine is stored in skeletal muscle and other organs also, i.e., lung and brain when circulated in blood.[13] Kidney and small intestine are responsible to maintain acid base balance by use of glutamine.[1]

Glutamine is formed glutamate and ammonium ion first catalyzed by glutaminase(GLS). Then glutamate is converted to α-ketoglutarate (αKT) by glutamate dehydrogenase. After that provide energy and macromolecular material sources by α-ketoglutarate (αKT) when it enters tricarboxylic acid(TCA) cycle.(In Figure 3.1)

Glutamine metabolism provides particularly carbon for acetyl COA, citrate production, lipogenesis, oxaloacetic acid(OAA) and nitrogen for purine, pyrimidine and DNA synthesis,[14] also reductive power NADPH to support cell proliferation.[15] The donation of the glutamine's amido nitrogen to nucleotides or hexosamines in addition to glutaminase(GLS) mediated by glutamine and fructose-6-phosphate amidotransferase could contribute a fraction of glutamate pool as well. During the cell cycle stage this might become more prominent characterized by transient increases in nucleotide biosynthesis or other activities.[16] For proper activation immune cells require high levels of extracellular glutamine. It has been reported that leukocytes metabolize glutamine at rates that are comparable to glucose utilization and even higher in some instances.[17]

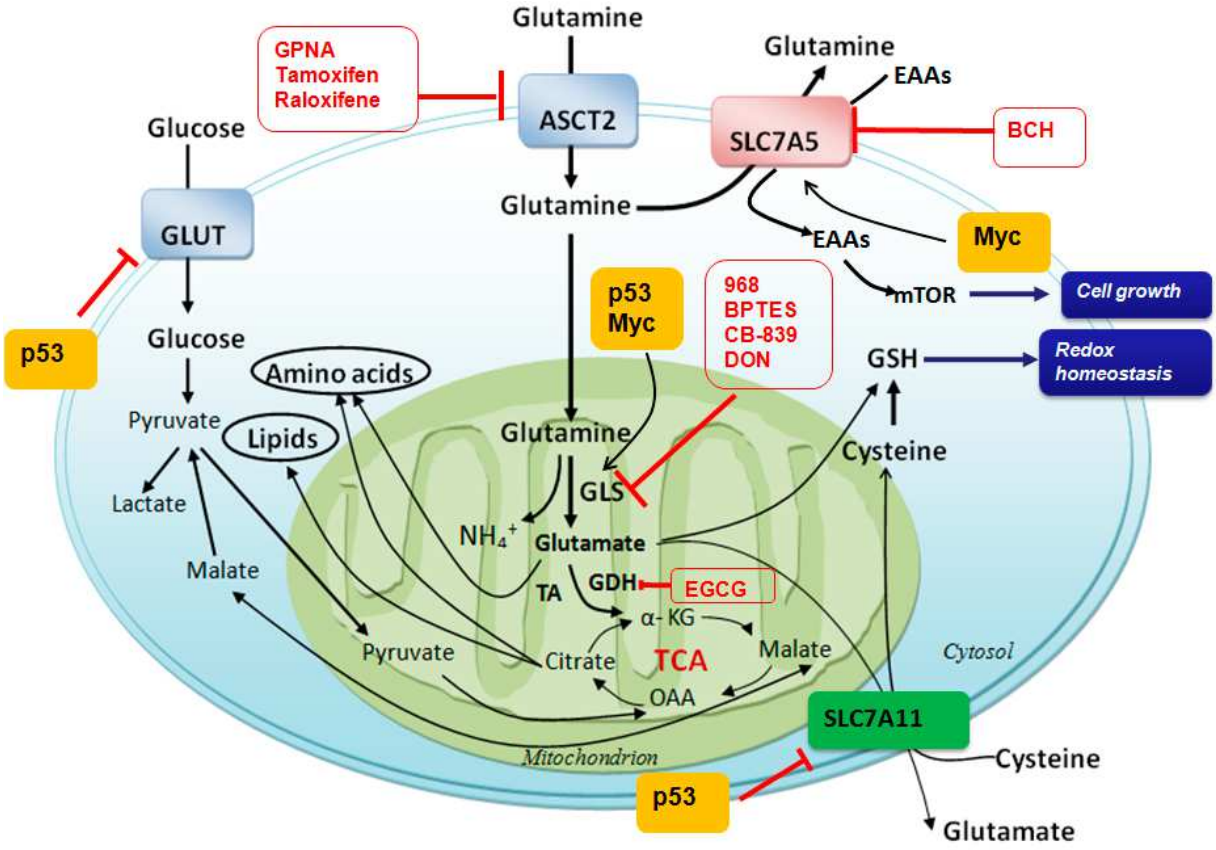


Figure 3.1. Glutamine metabolism and potent targets for cancer therapy. After transporting into cytosol by LAT1 (L-type amino acid transporters 1), ASCT2 (system ASC amino acid transporters 2) and other transporters, glutamine is catalyzed by glutaminase and converts to glutamate and ammonia. It then provides macromolecular material for ammonia acid and lipid syntheses. Glutamine is also used to exchange EAAs, which could activate mTOR and promote cell growth. Glutamate is also used to exchange extracellular cysteine for GSH production. GLS is a key enzyme for glutamine metabolism, which can be inhibited by several inhibitors including 968, BPTES and CB-839, accompanying with other inhibitors of glutamine metabolism are shown in red circle. GLS, glutaminase; GDH, glutamate dehydrogenase; TA, transaminase; OAA, oxaloacetate; BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; GPNA, γ-L-glutamylp-nitroanilide; EGCG, epigallocatechin gallate; EAAs, essential ammonia acids; mTOR, mammalian target of rapamycin; BPTES, bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide 3; 968, 5-(3-bromo-4-(dimethylamino) phenyl)-2,2-dimethyl-2,3,5,6-tetrahydrobenzo[a]phenanthridin-4(1H)-one;CB-839,N-(5-(4-(6-((2-(3-(trifluoromethoxy)phenyl)acetyl)amino)-3-pyridazinyl)butyl)-1,3,4-thiadiazol-2-yl)-2-pyridineacetamide. ⊥, inhibiting effect; bold black arrow, main metabolic pathway and transportation of glutamine; black arrow, metabolic pathways of glutamine and glucose.

3.4.2. Glutamine Metabolism in Cancer cells:

It is very clearly known that cancer cells in a dissipative way utilized glucose and produced ATP(adenosine-triphosphate) through aerobic glycolysis regardless of oxygen availability (Warburg effect).[18] To satisfy fast proliferation, cancer cells have to use another energy source-glutamine which produces ATP through glutamine-driven oxidative phosphorilation.[19] Due to continual loss of citrate from the TCA cycle in proliferating cells, especially in cancer cells, replenishment of TCA intermediates (anaplerosis) is necessary, and glutamine consumption is increased (Figure 3.2).

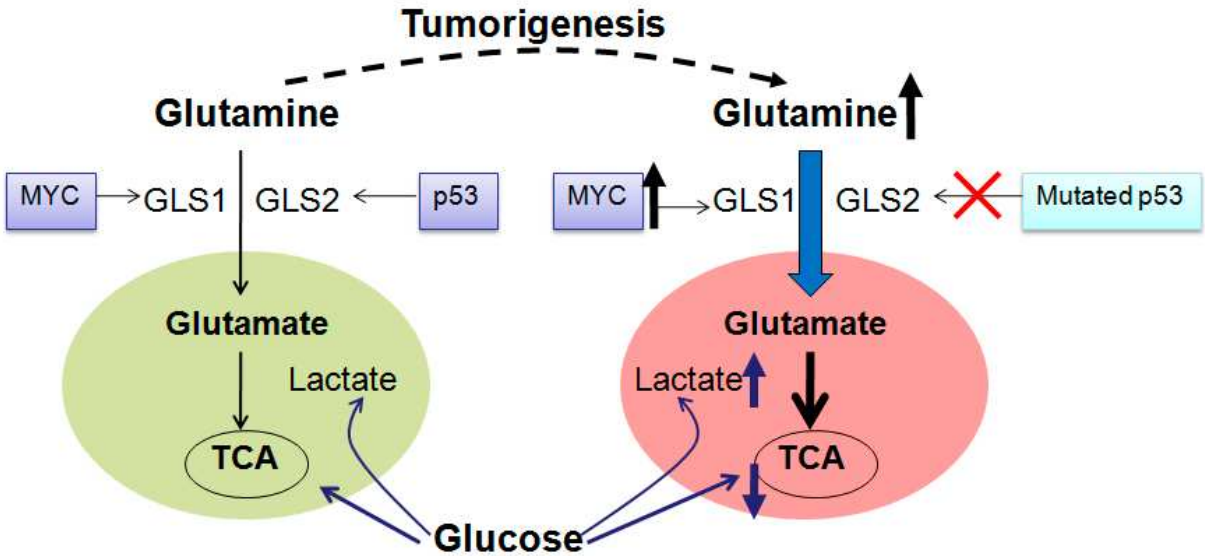


Figure 3.2. Glutamine consumption is increased in most tumors. During tumorigenesis, glucose derived lactate is increased, and at the same time, contribution of glucose to TCA is decreased. Accompanied with glucose metabolism change, glutamine metabolism is up-regulated to compensate energy and macromolecular for cell proliferation and growth. p53 is mutated, while MYC is overexpressed, which promotes glutamine metabolism by upregulating GLS1 activity during tumorigenesis. GLS1 is highly expressed in many tumors and promotes tumor proliferation. In contrast, GLS2 expression is reduced in some tumors. GLS, glutaminase; TCA, tricarboxylic acid cycle. Bold arrow, increased glutamine metabolism, decreased glucose metabolism and mutated MYC; dashed line, tumorigenesis procedure.

Higher consumption of glutamine in cancers is used for anabolic metabolism requirements which produces metabolic building blocks, such as nucleic acids, lipids and proteins),[20] and increases GSH production for cell death resistance. Also, the higher consumption of glutamine in cancers is used for EAAs (essential amino acids) exchanges that are required for cell growth and mTOR (mammalian target of rapamycin) activation which then initiates protein translation and cell growth (Figure 3.1).[21] Accompanied by increased glutamine consumption in most cancer cells, e.g., triple

negative breast cancer cell MX-1,[22] neuroblastoma cell[23] and human myeloid cell KU812F, GLS activity is also much higher than the normal cells. Elevated glutamine metabolism not only provides energy and substrates for cancer cells' growth and proliferation, but also makes glutamine a potent candidate in the cancer therapy (Figure 3.1).

In order to be well used by cells, glutamine has to across the plasma membrane and mitochondrial inner membrane and metabolizes in mitochondria[24] which is mediated by specific transporters. There are several different transport systems for amino acids to cross plasma membrane, e.g., Na+-dependent system A, Na+-dependent system N, Na+-dependent system ASC (alanine serine cystein) and Na+-independent system L .[25] The Na+-dependent system A maintains elevated intracellular concentrations of non-essential amino acids like glutamine and alanine that can be exchanged to essential amino acids through Na+-dependent system ASC and Na+-independent system L. The Na+-dependent system N can determine the extent of glutamine accumulation because it is able to mediate net glutamine efflux.[26] Glutamine transporters vary between normal and cancer cells. Glutamine transport in rat astrocytoma C6 cells and SK-N-SE cells is predominantly mediated by ASCT2 .[27,28] However, in normal rat astrocytes in situ, glutamine transport is mediated by a Na+-dependent system N, SN1.[27] During transformation, ASCT2 and LAT1 are the two main transporters for glutamine uptake.[28-31] It has been hypothesized that LAT1 provides the essential amino acids to enhance cancer cell growth via mTOR-stimulated translation and that ASCT2 maintains the cytoplasmic amino acid pool to drive LAT1 function . This cooperation of ASCT2 and LAT1 suppresses apoptosis and fuels the energy economy via net delivery of glutamine.[25]

However, not all cancers are addicted to glutamine. There are some cancer cells that can survive and proliferate by relying on glucose without glutamine. Cheng et al. have uncovered a compensatory relationship between GLS1 and PC (pyruvate carboxylase). Glucose-derived pyruvate serves as an anaplerotic precursor and thus, cells rely more on glucose in GLS1 silencing condition. The PC activity is induced in low-glutamine condition and is required for cells to escape glutamine addiction.[32] This compensatory relationship also needs to be taken into consideration in developing new cancer therapy that targets glutamine or glucose metabolism.

3.5. Glutamine promotes hallmarks of malignancy:

3.5.1.Deregulated energetic:

One hallmark of cancer cells is aberrant bioenergetics.[33] Glutamine's involvement in the pathways outlined above contributes to a phenotype conducive to energy formation, survival and growth. In addition to its role in mitochondrial metabolism, glutamine also suppresses expression of thioredoxin-interacting protein, a negative regulator of glucose uptake.[34] Thus, glutamine contributes to both of the energy-forming pathways in cancer cells: oxidative phosphorylation and glycolysis. Glutamine also

modulates hallmarks not traditionally thought to be metabolic, as outlined below. These interactions highlight the complex interplay between glutamine metabolism and many aspects of cell biology.

3.5.2. Sustaining proliferative signaling:

Pathological cancer cell growth relies on maintenance of proliferative signaling pathways with increased autonomy relative to non-malignant cells. Several lines of evidence argue that glutamine reinforces activity of these pathways. In some cancer cells, excess glutamine is exported in exchange for leucine and other essential amino acids. This exchange facilitates activation of the serine/threonine kinase mTOR, a major positive regulator of cell growth.[35] In addition, glutamine-derived nitrogen is a component of amino sugars, known as hexosamines that are used to glycosylate growth factor receptors and promote their localization to the cell surface. Disruption of hexosamine synthesis reduces the ability to initiate signaling pathways downstream of growth factors.[36]

3.5.3. Enabling replicative immortality:

Some aspects of glutamine metabolism oppose senescence and promote replicative immortality in cultured cells. In IMR90 lung fibroblasts, silencing either of two NADPH-generating isoforms of malic enzyme (ME1, ME2) rapidly induced senescence, while malic enzyme overexpression suppressed senescence. Both malic enzyme isoforms are repressed at the transcriptional level by p53 and contribute to enhanced levels of glutamine consumption and NADPH production in p53-deficient cells. The ability of p53-replete cells to resist senescence required the expression of ME1 and ME2 and silencing either enzyme reduced the growth of TP53+/+ and, to a lesser degree, TP53–/– tumors.[37] These observations position malic enzymes as potential therapeutic targets.

3.5.4. Resisting cell death:

Although many cancer cells require glutamine for survival, cells with enhanced expression of Myc oncoproteins are particularly sensitive to glutamine deprivation.[38] In these cells, glutamine deprivation induces depletion of TCA cycle intermediates, depression of ATP levels, delayed growth, diminished glutathione pools and apoptosis. The Myc drives glutamine uptake and catabolism by activating the expression of genes involved in glutamine metabolism including GLS and SLC1A5 which encodes the Na+-dependent amino acid transporter ASCT2.[39] Silencing GLS mimicked some of the effects of glutamine deprivation, including growth suppression in Myc-expressing cells and tumors.[32] The MYCN amplification occurs in 20%–25% of neuroblastomas and is correlated with poor outcome.[40] In cells with high N-Myc levels, glutamine deprivation triggered an ATF4-dependent induction of apoptosis that could be prevented by restoring downstream metabolites oxaloacetate and α-ketoglutarate.[23] In this model, pharmacological activation of ATF4(Activating transcription factor 4), inhibition of glutamine metabolic enzymes or combinations of these treatments mimicked the effects of glutamine deprivation in cells and suppressed growth of MYCN-amplified subcutaneous and transgenic tumors in mice.

The PKC isoform PKC-ζ also regulates glutamine metabolism. Loss of PKC-ζ enhances glutamine utilization and enables cells to survive glucose deprivation.[41] This effect requires flux of carbon and nitrogen atoms from glutamine into serine. The PKC-ζ reduces the expression of phosphoglycerate dehydrogenase an enzyme required for glutamine- dependent serine biosynthesis, and also phosphorylates and inactivates this enzyme. Thus, PKC-ζ loss which promotes intestinal tumorigenesis in mice, enables cells to alter glutamine metabolism in response to nutrient stress.

3.5.5. Invasion and metastasis:

Loss of the epithelial cell-cell adhesion molecule E-cadherin is a component of the epithelial-mesenchymal transition and is sufficient to induce migration, invasion, and tumor progression.[42,43] Addiction to glutamine may oppose this process because glutamine favors stabilization of tight junctions in some cells.[44] Furthermore, the selection of breast cancer cells with the ability to grow without glutamine yielded highly adaptable subpopulations with enhanced mesenchymal marker expression and improved capacity for anchorage-independent growth, therapeutic resistance, and metastasis in vivo.[45] It is unknown whether this result reflects a primary role for glutamine in suppressing these markers of aggressiveness in breast cancer or whether prolonged glutamine deprivation selects for cells with enhanced fitness across a number of phenotypes.

3.6. Glutamine Metabolism Regulation in Cancers:

In cancers, glutamine metabolism is highly regulated by several factors such as MYC, p53, Ras and HIF (hypoxia-inducible factor). This modulation is maintaining cancer growth and also is one of the reasons for carcinogenesis. Which are :

3.6.1. MYC in Glutamine Metabolism in Cancers:

The proto-oncogene MYC regulates about 15% of genes in genomes from flies to humans.[46] It includes N-MYC, C-MYC and L-MYC those are deregulated, mutated or amplified in most human tumors.[47-49] They can be activated by mitogenic signals and drive cell proliferation. The C-MYC is broadly deregulated in many human cancers, N-MYC expression is more restricted to neural cancers and L-MYC is predominantly found in small cell lung cancer.[23]

In some cancers, MYC amplification is involved in glutamine addiction (Figure 3.2). Glutamine addiction is correlated to MYC-induced redirection of glucose carbon away from mitochondria that is a result of LDHA (lactate dehydrogenase) activation.[21] More specifically, MYC-induction leads to the diversion of glucose-derived pyruvate away from mitochondria and is converted to lactate.[50] As a result, MYC-transformed cells become dependent on glutamine anapleurosis for maintaining the mitochondrial integrity and TCA cycle function. Also, MYC is likely to increase activities of GLS1[39] (as shown in the next part) and glutamine synthetase.[51] In addition, MYC probably binds to the promoter elements of glutamine transporters which is associated with enhanced levels of glutamine transporters,

e.g., SLC7A5 (solute carrier family 7 member 5, LAT1) and ASCT2 (Figure 3.1).[39,50] The N-MYC overexpression stimulates mRNA and protein expression of the catalytic subunit of GCL (glutamate-cysteine ligase) and causes rate-limiting step in GSH biosynthesis which increases GSH level and provides resistance to oxidative damage.[52] Therefore, targeting MYC can provide a therapeutic window for cancers that have MYC amplification. Down-regulation of N-MYC expression has been proved to induce apoptosis, and to decrease proliferation and/or neuronal differentiation in neuroblastoma cells in vitro.[53] Similar results are also observed in lymphoma, leukemia, osteosarcoma, hepatocellular carcinoma, squamous carcinoma, and pancreatic carcinoma.[54]

3.6.2. p53 in Glutamine Metabolism in Cancers:

As one of the cell fate determinants, p53 gene is found to be mutated or dismantled in most human cancers (Figure 2).[55] It is widely accepted that p53 is a tumor suppressor gene which is able to induce cell cycle arrest and apoptosis under DNA damage, hypoxia or oncogene activation conditions. Normally, p53 gene is located in the nuclears.[56] It translocates to cytosol and binds to its cytosolic MDM2 after translation, and this binding inhibits p53 activation.

The GLS2 has been proved to be a target of p53. By up-regulating GLS2 expression (Figure 3.1), p53 increases GSH levels and reduces ROS levels which then inhibits tumorigenesis. Unfortunately, p53 is mutated in many cancers which indicates loss of functions. Apart from working on GLS2, p53 is recently reported to repress expression of SLC7A11 (Figure 3.1), a key component of the cysteine/glutamate antiporter.[57] The SLC7A11 mediates exchange of extracellular cysteine to intracellular glutamate [58], and is overexpressed in several human cancers.[57] Also, p53 can repress GLUT1 (glucose transporters) and GLUT4 and inhibits PI3K (phosphatidylinositol-3 kinase)–AKT (protein kinase B) and mTOR pathways. These effects of p53 result in cell growth repression and then reverse the cancer phenotype.[59] Based on its importantly inhibitive role in cancers, it is particularly interesting in trying to restore or increase p53 activity in p53 mutated or loss of function cancers.

3.6.3. Ras in Glutamine Metabolism in Cancers:

Oncogenic Ras proteins are identified in 25% of human cancers and are correlated to metabolic alterations. Ras increases utilization of the carbon backbone and amino-nitrogen moieties of glutamine, and promotes glucose consumption.[60] Ras-driven cancers are able to satisfy their nutritional needs through activation of fluid-phase endocytic nutrient uptake and promotes angiogenesis to increase the tumor blood supply. It has been revealed that glutamine is the major carbon source for the TCA cycle when Ras is activated.[61] The reprogramming of glutamine metabolism is mediated by oncogenic K-Ras in human pancreatic ductal adenocarcinoma. K-Ras can repress glutamate dehydrogenase expression and

increase aspartate transaminase expression.[62] At the same time, glutamine depletion can induce proliferation arrest of K-Ras-transformed cells.[63]

3.6.4. Hypoxia-Inducible Factor (HIF) in Glutamine Metabolism in Cancers:

HIF-1, a heterodimer, is composed of HIF-1α and HIF-1β subunits. The expression and activity of the HIF-1α subunit are tightly regulated by cellular oxygen concentration. The expression levels of α subunit are increased during hypoxia whereas the β subunit is constitutively expressed.[64]

The HIF-1 plays a key role in reprogramming cancer metabolism by activating transcription of many genes that encode glucose transporters as well as glycolytic enzymes and it also promotes angiogenesis. The HIF-1 is both necessary and sufficient for reducing mitochondrial oxygen consumption in hypoxia by inducing PDK1 (pyruvate dehydrogenase kinase 1).[65] In this way, cancers can survival under hypoxia. However, if PDK1 activity is inhibited, continued mitochondrial respiration and resulting oxidative stress will induce cell death.

Solid tumors are often poorly vascularized and contain region hypoxia. This special tumor microenvironment enhances HIF-1α over-expression in the majority of human cancers and their metastases, where HIF-1α induces gene expression to promote survival.[66] Under hypoxia, reduced F1Fo ATPase activity can increase phosphate concentration in mitochondria. Increased phosphate promotes GAC (elongated kidney glutaminase variant)-based glutaminase activity which enhances glutaminolysis to provide increased metabolic and biosynthetic needs for conferring selective advantage to malignant cells.[67] The above-mentioned metabolic regulation makes malignant cells more susceptible to GLS inhibitor and hence can be targeted for cancer therapy. During hypoxia, there is a decrease in glucose-derived citrate due to decreased pyruvate dehydrogenase activity, and an increase in α-KG levels caused by reduced α-KGDH (ketoglutarate dehydrogenase) activity. These changes drive the reverse reaction at IDH (isocitrate dehydrogenase)[68] and then increase citrate production, and finally make cells rely on reductive glutamine metabolism in the lipid synthesis. The LKB1 (liver kinase B 1) is a serine/threonine kinase and is often inactivated in human cancer. It has been demonstrated that loss of LKB1 makes cancer cells rely on HIF-1α in the ATP supply which induces an increase in glycolysis and glutamine consumption. Reasonably, knockdown of HIF-1α in cells without LKB1 promotes a decrease in glutamine consumption by these cells.[69]

3.7. Glutamic acid as anticancer agent:

L-Glutamic acid is converted into L-glutamine by L-glutamine synthetase. L-Glutamine biosynthesize purines and pyrimidines by contributing 3- and 9-nitrogen groups of purine bases, 2-amino group of guanine, 3-nitrogen group and amino group of cytosine which are the bases of DNA and RNA.[70] L-Glutamine cannot be synthesized in neoplastic cells due to the lower reactivity of L-glutamine synthetase. Thus, an antagonist of this enzyme can interfere with the metabolic process of L-glutamine and act as

anticancer agents. Patients of cancer often develop glutamine depletion in the muscles due to uptake by tumors and chronic protein metabolism. On the basis of these, it can be assumed that structural variants of glutamine might possess possible antitumor activity.

L-Glutamic acid-γ-(4-hydroxyanilide) is isolated from mushroom Agaricus bisporous. It acts as a growth regulatory substance for inhibiting the B16 melanoma cells in culture. Azaserine and 6-diaza-5-oxo-L-norleucine antagonized the metabolic process involving L-glutamine and exhibited antitumor activity in animal models.[71] An aryl amide derivative of L-threo-γ-hydroxy glutamic acid which is isolated from Justica ghiesbreghtiana is active against various tumors. The synthetic amides of L-glutamic acid exhibit activity against Ehrlich Ascites carcinoma cells. Four N-(benzenesulfonyl)-L-glutamic acid bis(p-substituted phenylhydrazides) have anticancer activity against PC-3 prostate cancer and in COLO-205 colon cancer. L-Glutamic acid-γ-monohydroxamate (GAH) demonstrated complete cytotoxicity against L-1210 cells in the culture and marked antitumoral activity in vivo against L-1210 leukemia and B-16 melanoma. Glutamate receptor is another important player in hippocampal long-term potentiation and memory. Glutamic acid, a glutamate receptor agonist enhances retention of memory.[72] Glutamic acid is also useful in lowering blood pressure. According to a study by the Imperial College of London, people who consume more glutamic acid have low blood pressure than those who consume less. When the glutamine importer SLC1A5 is impaired then the uptake of essential amino acids is also impaired and without the aid of essential amino acids rapamycin-sensitive mTORC1 is not activated. mTORC1 is responsible for the regulation of cell growth, protein translation and plays an important role in inhibiting macro autophagy.[73] That means if mTORC1 is inactivated then there will be no cell growth and no protein translation. In glioblastoma cells, metabolism of glutamine provides the bulk of oxaloacetic acid (OAA) cellular pool.[15] This OAA is one of the substrates in mitochondria that leads to the synthesis of many essential biological macromolecules like cholesterol. Hence glutamine is the primary substrate in cancer cells that provides precursor molecules to mitochondria for anaplerosis (replenishment of the carbon pool). c-MYC (Myc), a DNA transcription factor regulates three out of the five steps of purine and pyrimidine synthesis at oncogenic level. It also promotes glutaminolysis and this catabolism of glutamine leads to the larger amount of carbon in the cell, which allows the cell to produce more NADPH.[3] Since cancer cells depend on glutamine lack of glutamine, can lead to the death of cancer cells. But as it is also required for some other essential functions in the body such as in the brain therefore that treatment should be adopted which can reduce the ability of the cell to uptake glutamine by targeting Myc and other proteins that are responsible for transporting glutamine into the cell. L-γ- Glutamyl-p-nitroanilide (GPNA) which inhibits SLC1A5 and BCH (2-aminobicyclo-(2,2,1)heptanes carboxylic acid) which blocks mTORC1[73] and that treatment which reprograms the mitochondria so that it no longer

depends on glutamine can also be effective, e.g. amino-oxyacetic acid (AOA) is used because it is a transaminase inhibitor.

3.8. Glutamic acid as conjugates with anticancer drug:

Glutamic acid is used as a conjugate because it increases the efficacy of anticancer drug and decreases its toxicity toward normal cells. Polyglutamic acid is biodegradable, edible and nontoxic toward humans.[74]

3.8.1. Conjugate with All-trans retinoic acid (ATRA):

ATRA is an active metabolite of vitamin A. It is used in the treatment of acute promyelocytic leukemia and myelodysplastic syndrome. It has a slow dissolution rate and low bioavailability. Therefore to obtain ATRA with better solubility, transportation and bioavailability, derivatives of ATRA containing glutamic acid or its sodium salt were synthesized. The two derivatives of ATRA, RAE and RAENa, exhibited improved aqueous solubility and were more effective in mice bearing S(180) tumors.[72]

3.8.2. Conjugate with paclitaxel

Conjugation of paclitaxel and the water-soluble polyglutamate is known as poly (L-glutamic acid)-paclitaxel (PG-TXL). Observations showed that PG-TXL has more antitumor activity than free paclitaxel. PG-TXL exerts its anticancer activity by the continuous release of free paclitaxel into cells.[75]

3.8.3. Conjugate with cisplatin:

Cisplatin [cis-dichlorodiammineplatinum (II), CDDP] is combined with γ-poly(α,L-glutamic acid) (γ-PGA) to form γ-poly(α,L-glutamic acid)-cis-dichlorodiammineplatinum (γ-PGA-CDDP) which is water soluble. γ-PGA–cisplastin conjugate effectively inhibits human breast tumor cells xenografted into nude mice. It also reduces the toxic side effects associated with the use of free CDDP and also produced desirable pharmacokinetics and enhanced antitumor activity [76] (Figure 3.3).

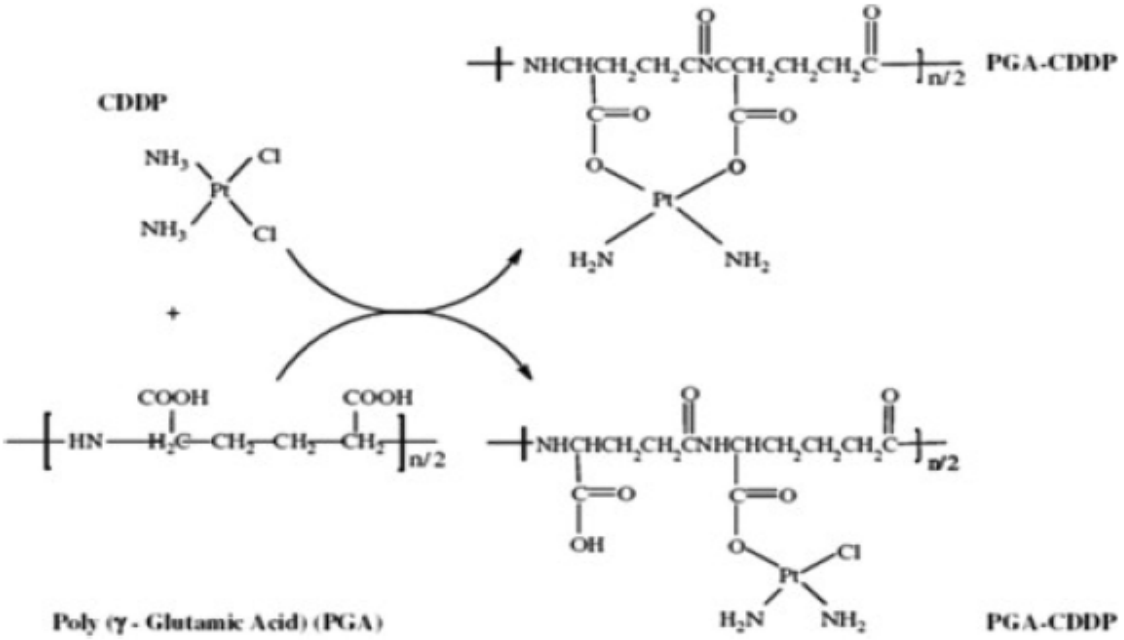


Figure 3.3. Chemical structures of CDDP, γ-PGA, and PGA–CDDP conjugates.

3.8.4. Conjugate with curcumin:

Curcumin [diferuloylmethane; 1,7-bis-(4-hydroxyl-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the major pigmentary component of turmeric (Curcuma longa). Curcumin with naphthaloylglutamoylchloride forms 4,40-(di-o-glutamoyl)-curcumin. According to a study curcumindiglutamoyl derivative was found to be more potent against cancer cell lines, HeLa (cervical cancer) and KB (oral cancer) than other derivatives. It is due to the activation of caspases which is facilitated due to accumulation and better stability of diglutamiccurcumin.[77]

3.8.5. Conjugate with 20(s)-camptothecin (CPT)

Due to the instability of active lactone the therapeutic efficacy of 20(s)-camptothecin (CPT) is limited in humans. By binding one molecule of a drug via the γ-carboxylic acid of each monomeric subunit of poly-(L-glutamic acid) (PGA) it leads to the stability of lactone. Linking of CPT to a high molecular weight anionic polymer that is PGA enhances solubility and improves distribution to the tumor through enhanced permeability and retention (EPR effect)[78].

3.8.6. Conjugate with N-(4-hydroxyphenyl)retinamide (4HPR):

Several studies have indicated that N-(4-hydroxyphenyl)retinamide (4HPR) treatment is associated with the inhibition of angiogenesis and a decreased vascular response in vitro and in vivo. 4HPR was bound to a synthetic polyamino acid, poly(L-glutamic acid) (PG). PG- 4HPR was evaluated for its release kinetics and in vitro anti-proliferative and in vivo antitumor activities against ovarian cancer cell lines. It was confirmed that treatments with both 4HPR and PG-4HPR decreased the expression of pre-angiogenic

factor VEGF in SKOV3 tumors. Invivo, PG-4HPR demonstrated significantly enhanced antitumor activities compared to 4HPR in both early treatment and later treatment protocols. Treatments with PG-4HPR suppressed the expression of VEGF and reduced blood flow into the tumor.[79]

3.9. Derivatives of glutamic acid as anticancer agent:

3.9.1. Aminopteroylglutamic acid or pteroyl-l-glutamic acid:

Aminopterin (4-aminopteroic acid), a 4-amino analog of folic acid, is an antineoplastic drug with immunosuppressive properties used in chemotherapy.[80] Folate is involved in DNA synthesis and methylation which may reduce breast cancer risk, particularly among women with greater alcohol consumption. High intake of folate may reduce the risk of colon cancer, but the dosage and duration relations and the impact of diet compared with supplementary sources are not well understood.[81] Folate intake decreased the risk of pancreatic cancer in women but not in men.

3.9.2. Methotrexate:

Chemically methotrexate is N-[4-[[(2,4-diamino-6-pteridinyl) methyl] methylamino] benzoyl]-Lglutamic acid. Methotrexate tablets are used alone or in combination with other anticancer agents in the treatment of breast cancer, epidermoid cancers of the head and neck, advanced mycosis fungoides (cutaneous T-cell lymphoma), and lung cancer, particularly squamous cell and small cell types. Methotrexate tablets are also used in combination with other chemotherapeutic agents in the treatment of advanced stage non-Hodgkin's lymphomas. It reduces dihydrofolates to tetrahydrofolates by the help of enzyme dihydrofolic acid reductase which inhibits the synthesis of purines.[82]

3.9.3. L-Theanine:

Chemically L-theanine is γ-ethylamino-L-glutamic acid. Limited studies evaluate the effects of L-theanine in the prevention of cancer. The observed anticancer effects are largely attributed to the catechins found in tea, while action on tumors may be due to an enhanced immune response.[83]

3.9.4. Thalidomide:

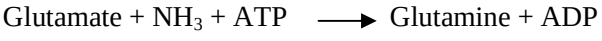
It is a chemotherapeutic agent used against multiple myeloma, myelodysplastic syndrome, leprosy etc. It acts by inhibiting VEGF, TNF-α, GI growth factor, proliferation of NK cells and stimulation of T-cells.

3.10. Glutamate and its metabolism enzymes:

The carboxylate anions and salts of glutamic acid are known as glutamates. It is also important for metabolism.

3.10.1. Glutamine synthetase:

It acts as a catalyst in this reaction,



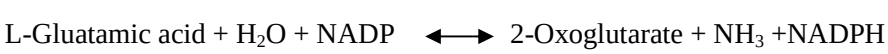
The enzyme from mammalian brain has two important functions: assimilation of ammonia and biosynthesis of glutamine.[84]

3.10.2. Glutaminase:

Glutaminase catalyzes the reaction of glutamine catabolism to produce glutamate and ammonia. Its activity can be observed both in neurones and astroglial cells, but glutaminase is more active in neurons, predominantly in mitochondria. The enzyme becomes free from the inhibitory influence of glutamate only when the concentration of glutamic acid falls below normal values.[85]

3.10.3. Glutamate dehydrogenase:

Glutamate dehydrogenase (GDH) catalyzes a reversible reaction:



The GDH reaction is reversible but equilibrium is shifted closer to direct reaction (i.e. to synthesis of glutamic acid).[86] Consequently, GDH in brain takes part in the synthesis of glutamate from 2-oxoglutaric acid, more than in oxidation of the amino acid. This is the way of continuous maintenance for changing of free ammonia into amino nitrogen of amino acids.

3.10.4. Aspartate transaminase or glutamate oxaloacetate transaminase:

Aspartate transaminase catalyzes the reversible transfer of amino group to 2-oxoglutarate, as a result oxaloacetate and glutamate are formed. This is the most active transaminase in the brain.[87] Oxaloacetate is quickly transformed into malic acid which enters from the cytoplasm into mitochondria. In this way the aspartate-malate bypath for transfer redox equivalents from the cytosol to mitochondria is formed. This bypath is the main way for transferring redox equivalents to mitochondria in neurons. Influx of aspartate through the mitochondrial membrane is bound with an efflux of a glutamate in such a way influx of a malate is bound with the efflux of 2-oxoglutarate, too.

3.10.5. Glutamic acid decarboxylase:

Glutamic acid decarboxylase (GDA) catalyzes the separation of a carboxylic group from glutamate with the formation of gamma-aminobutyric acid (GABA). When GAD acts on glutamate which has an excitatory influence on neurons, then GABA is formed which is a main inhibitory neurotransmitter in the brain.[88]

3.11. Pharmacological uses of glutamic acid:

3.11.1. Fuel:

Glutamic acid takes part in more metabolic reactions than any other amino acid. It is a source of glucose which is the chief source of fuel. It maintains the normal blood glucose level and also the acidity. It serves

as a source of fuel for the intestinal epithelium. Glutamine increases the body's secretion of human growth hormone (HGH). It also plays a major role in removing excess ammonia from the bloodstream.

3.11.2. Muscle and other cell components:

Glutamine is present in more than three-fifths of the human muscle tissue. It synthesizes both DNA and RNA and is essential for the synthesis of all proteins. By stimulating the release of the anabolic hormone HGH, it plays an indirect role in building the skeletal muscle. Glutamine also protects the integrity of the gastrointestinal tract when ulcers, chemotherapy agents or non-steroidal anti-inflammatory drugs are present.

3.11.3. Immune function:

Glutamine is very essential for proper immune function. It is required for the proliferation of lymphocytes and cytokines. It increases the efficiency of macrophages, large immune cells which engulf and degrade foreign substances ranging from microbes to inorganic molecules. In many situations in which the immune function is compromised, for example, severe burns, sepsis or when athletes overexert themselves, glutamine depletion is at least partly responsible for the immune suppression. The administration of glutamine to patients receiving bone marrow transplants results in a lower incidence of post-operative infections and a shorter stay in the hospital.

3.11.4. Neurotransmitter:

It is an important excitatory neurotransmitter. It helps in the transportation of potassium across the blood–brain barrier. It also shows promise in the future treatment of neurological conditions, ulcers, hypoglycemic coma, muscular dystrophy, epilepsy, Parkinson's and mental retardation.

3.11.5. Other uses:

The other uses of Glutamic acid are-

3.11.5.a. As surfactants:

It is hydrophilic by nature and can combine with hydrophobic fatty acids easily to form molecules with both water-soluble and water repelling portions that can be used as surfactants. There are many applications of GA derivatives as surfactants, especially due to its lack of harmful effects to skin and their general smooth appearance. It is very much favored by the cosmetic, moisture containing hair shampoo product manufacturers.

3.11.5.b. As Chelating agents:

GA has two carboxylic groups, which can form chelates with many metal cations. Such chelating reaction is useful in the removal of heavy-metal contaminants in the wastewater treatment processes.

3.11.5.c. As flavor enhancer:

Monosodium glutamate (MSG), the single largest amino acid product, has been used as a flavor enhancer throughout the world for the past forty years. MSG is used worldwide in huge quantities as a flavor

enhancer in foods. MSG is known to produce a unique taste sensation termed 'UMAMI' the fifth taste, i.e. savory or brothy taste present in tomatoes and cheese. Free glutamate content is said to increase during the process of natural ripening and brings about a fuller taste in many foods, the basis behind is not known.

3.11.5.d. As buffer:

GA is an amphopteric substance that contains both acidic and basic functional groups and thus a natural buffer by itself.

3.11.5.e. In Agriculture:

GA is one of the major amino acids in plant proteins and plays a role of the major nitrogen storage for plants. That is why GA is often one of the more prominent ingredients in many plant growth supplements. Besides, GA is vital in the nitrogen metabolism in plants.

3.11.5.f. In culture medium:

GA happens to be one of the main components in the cell wall of Gram-positive bacteria. It is also one of the most essential amino acids for other microorganisms to grow on. In most cases, it is often necessary to add GA into culture media to effect normal growth.

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4. SYNTHETIC CHEMISTRY OF ISOGLUTAMINE DERIVATIVES

4.1. Introduction:

Although there have been great advances in the detection and treatment of cancer, it remains one of the greatest medical challenges, with the incidence of some malignancies continuing to increase .[1] For many tumor types, established treatments such as cytotoxic chemotherapy and radiotherapy provide only transient therapeutic benefits despite severe side effects.[2] Therefore, the need for better treatments has stimulated research to develop new efficient chemotherapeutic agents for management of cancer.

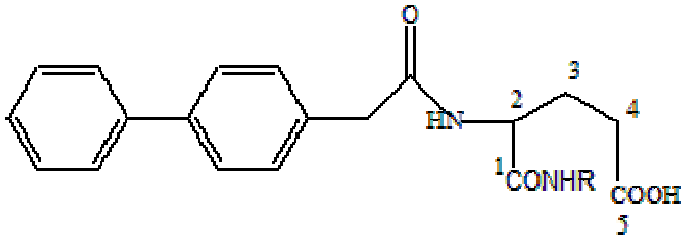
It was observed that tumors assimilate not only the nitrogen from the diet but also the nitrogen from host proteins, raising the concept of tumors as “nitrogen traps”, actively competing with the host for nitrogen compounds.[3] The causes of cancer are diverse, complex, and only partially understood.

Glutamine is one of the major substrates, if not the major substrate, for the energy metabolism of rapidly growing tumor cells . Glutamic acid (2-amino pentanedioic acid) plays an important role in the biosynthesis of purine and pyrimidine bases of DNA and RNA .[4] Glutamine acts as a nitrogen donor in the nucleotide and amino acid biosynthesis, secondly glutamine helps in the uptake of essential amino acid and it maintains the activation of TOR kinase. For normal maintenance of cells, metabolism of glutamic acid to L-glutamine by L-glutamine synthetase is essential. The synthesis of L-glutamine is hindered in neoplastic cells due to lower reactivity of L-glutamine synthetase. Thus, antagonists of this enzyme can interfere with the metabolic role of L-glutamine and act as anti-cancer agents.[5]

With the perspective of developing better active anticancer agents, various analogs of 1-N-substituted-2-N-(substituted biphenylacetyl)-L (+)-isoglutamine were synthesized.

4.2. Synthesis of Isoglutamine Derivatives:

In our laboratory there are 14 compounds of Isoglutamine analogue are synthesized.



General structure of 1-N-substituted-2-N-(substituted biphenylacetyl)-L(+)- isoglutamine

The synthesis of 1-N-substituted-2-N-(substituted biphenylacetyl)-L(+)-isoglutamine analogues were started with substituted biphenylacetic acid . The first step of synthesis includes acid chloride formation of substituted biphenylacetic acid using thionyl chloride to yield the substituted biphenylacetyl chloride. In the second step, the substituted biphenylacetyl chlorides is treated with L(+)-glutamic acid in the alkaline medium maintained by 2N NaOH at 85-90°C by applying Schotten Baumann reaction to produce the 2-N-(substituted biphenylacetyl) L(+)glutamic acid. The medium was kept alkaline to remove the HCl produced during the condensation reaction. The third step includes the amination of the 2-N-(substituted biphenylacetyl) glutamic acid to yield the corresponding 1-N-substituted-2-N-(substituted biphenylacetyl)-L(+)- isoglutamines.

4.3. The steps involve in this synthesis process:

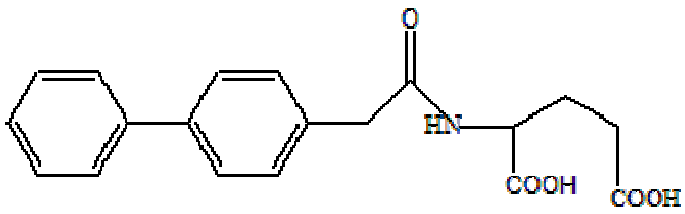
Step 1: Synthesis of substituted biphenylacetylchloride:

A mixture of substituted biphenyl acetic acid (0.1 mole) and benzene (50-60 ml) was taken in a 250 ml round bottomed flask. The flask was fitted with a reflux condenser attached with a calcium chloride drying tube and dropping funnel. Thionyl chloride (0.4-0.5 mole) was added dropwise from the dropping funnel to the round bottomed flask under anhydrous condition. The reaction mixture was refluxed for 3-4 hrs until HCl gas emission stops. The evolution of HCl gas was checked by congo red paper. The excess of thionyl chloride was removed by distillation with three 50 ml portions of dry benzene to obtain the product individually.

Step 2: Synthesis of 2-N-(substituted biphenylacetyl)-L(+)-glutamic acid:

L(+)-glutamic acid (0.1 mole) was taken in a dry 250 ml conical flask. 2N sodium hydroxide solution was added (to make the solution slight alkaline) slowly to the conical flask to dissolve all glutamic acid. The pink coloration of the phenolphthalein paper, when a drop of

reaction mixture was touched, indicates slightly alkaline nature of the mixture. The reaction mixture was stirred on the thermostatically controlled magnetic stirrer with temperature maintained at 85-90°C. Biphenylacetyl chloride was added to the reaction mixture in small portions with constant stirring. To keep the reaction mixture alkaline throughout the reaction, time to time, small amount of sodium hydroxide solution (2N) was added to the reaction mixture. The reaction was continued until a clear homogeneous solution was obtained. After the reaction was over, it was allowed to cool at room temperature. Filtration was carried out to separate insoluble solid matter if present in the solution mixture. The filtrate was acidified with hydrochloric acid solution. The acidified mixture was extracted with three 50ml portions of ethyl acetate. Ethyl acetate extract was washed with 10ml portion ice cold water (or chilled brine solution) for three times and dried overnight with anhydrous sodium sulphate. In the next day ethyl acetate was distilled off to get the desired crude product which was used subsequently without purification.



Biphenyl acetyl Glutamic acid(Diacid)

COMPOUND	MOLECULAR WEIGHT	MOLECULAR FORMULA	% YIELD	MELTING POINT
Biphenyl acetyl glutamic acid	334	$C_{19}O_5N_1H_{19}$	82	135-137

Step 3:Synthesis of 1-N-substituted-2-N-(substituted biphenylacetyl)-L(+) isoglutamines:

A mixture of 2-N-(substituted biphenylacetyl)-L(+) glutamic acid (0.01 mole) and 50-60 ml of chloroform was taken in a 250 ml of flat bottomed flask. The reaction mixture was stirred on the magnetic stirrer with temperature maintained at 0-5°C using salt ice mixture under anhydrous condition. N,N Dicyclohexylcarbidiimide (DCC) (0.01 mole) dissolved in 30-40 ml chloroform was added slowly and gradually to the reaction mixture with continuous stirring. After 1hr, amine (0.01 mole) was added to the reaction mixture with continuous stirring. The stirring was contined for 5-6 hrs. After the reaction was over, the reaction mixture was kept in the fridge for overnight. The solution was filtered and both the solid part

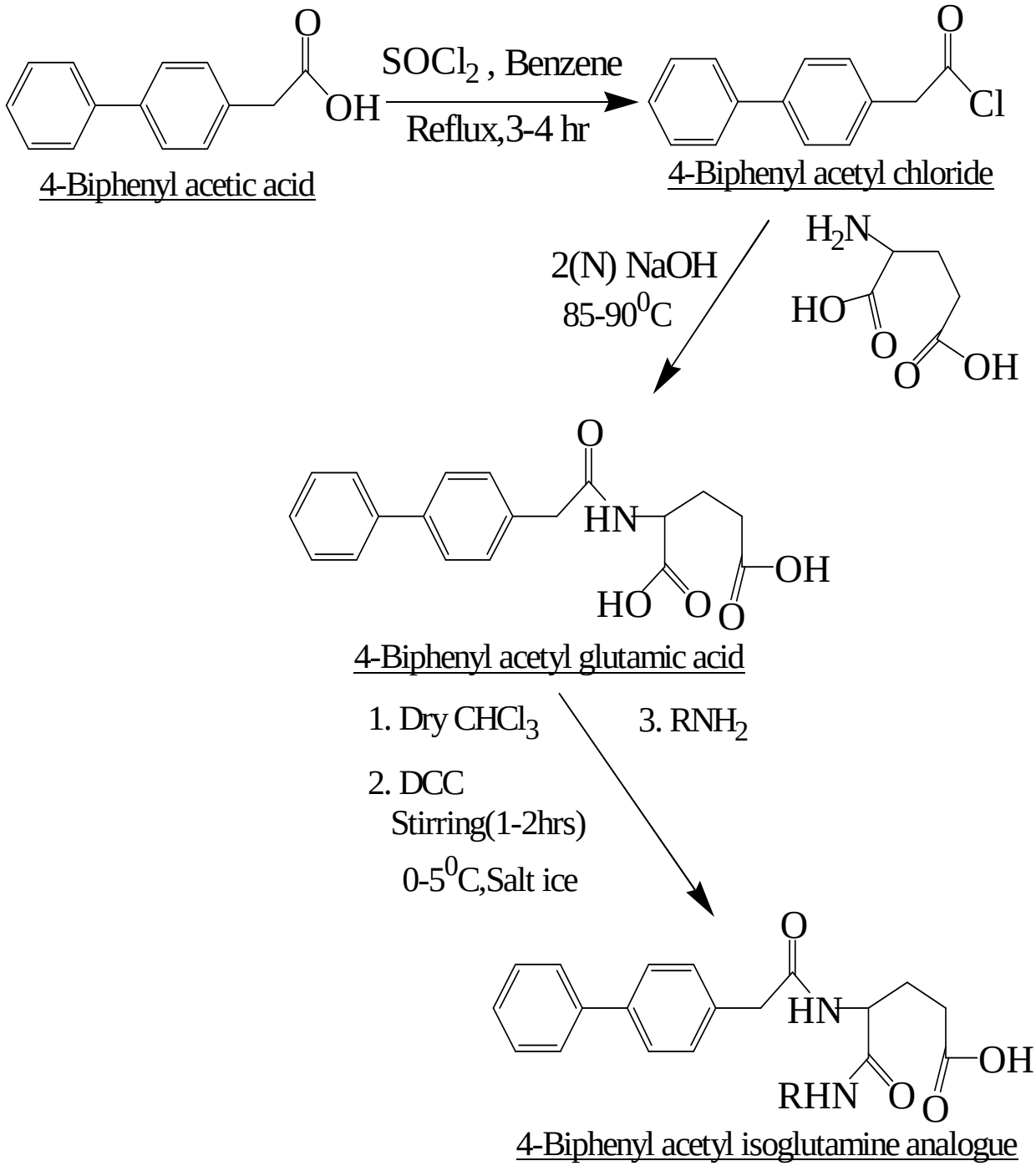
(DCU) and the liquid part (chloroform) was extracted with 1N sodium carbonate (Na_2CO_3) solution. The sodium carbonate part was acidified with HCl in cold condition. The solid product was precipitated and the solution was cooled for 1-2 hr to allow more precipitation. The precipitate was filtered, washed several time with distilled water and dried under suction.

4.4. Recrystallisation of 1-N-substituted-2-N-(substituted biphenylacetyl)-L(+)-isoglutamines:

The product was taken in a conical flask and 20-30 ml of ethyl alcohol was added. It was heated. After dissolving the product, it started boiling. At the time of boiling, small amount of charcoal was added and it was heated for more 20-30 seconds. It was filtered at once by using a filter paper. The solution was collected in a clean conical flask and kept in refrigerator. After 1-3 days, when the sufficient product was precipitated in conical flask, the product was collected by filtering. The recrystallised product was dried**.**

4.5. Schematic Pathway of Synthesis of 1-N-substituted-2-N-(substituted biphenylacetyl)-L(+)- isoglutamines derivatives:

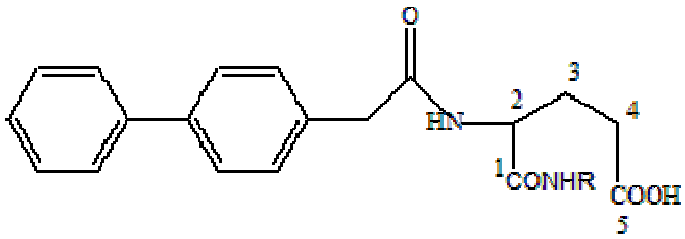
Schematic Pathway of Synthesis of 1-N-substituted-2-N-(substituted biphenylacetyl)-L(+)-isoglutamines derivatives shown below:



4.6. List of synthesized 1-N-substituted-2-N-(substituted biphenylacetyl)-L(+)-isoglutamine derivatives and their Physical data:

The Isoglutamine structure amine derivatives (i.e R) are listed below-

Sl. Nos.	Amine derivatives (R) name list	Molecular weight
1	n-butyl(R_1)	73.14
2	i-butyl(R_2)	73.14
3	Phenyl(R_3)	93.13
4	2-Cl Benzyl(R_4)	141.60
5	4-F Benzyl(R_5)	125.15
6	n- pentyl(R_6)	87.16
7	Benzyl(R_7)	107.16
8	4-Nitrobenzyl(R_8)	152.12
9	n-hexyl(R_9)	101.19
10	3,5-Bis di(trifluoromethyl) Benzyl (R_{10})	243.51
11	O-phenylene diamine(R_{11})	108.14
12	2-amino benzimidazole(R_{12})	133.15
13	4-amino biphenyl(R_{13})	169.23
14	2-amino benzoxazole(R_{14})	134.14



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Physical data of synthesized 1-N-substituted-2-N-(substituted biphenylacetyl)-L(+)-isoglutamine derivatives are listed below-

Sl. No.	COMPOUNDS	Mol. formula	Mol.Wt.	M.P	% Yield
1	1-N-isobutyl-2-N-(4-biphenylacetyl)-L(+)-isoglutamine	C23H29N2O4	396.14	156-158	49.53
2	1-N-phenyl-2-N-(4-biphenylacetyl)-L(+)-isoglutamine	C25H25N2O4	396.14	160-162	48.28
3	1-N-(4-Fluorobenzyl)-2-N-(4-biphenylacetyl)-L(+)-isoglutamine	FC26H26N2O4	416.13	152-154	16.52
4	1-N-(n-hexyl)-2-N-(4-biphenylacetyl)-L(+)-isoglutamine	C25H33N2O4	464.60	148-150	15.00
5	1-N-3,5,-Bisdi(trifluoromethyl)benzyl-2-N-(4-biphenylacetyl)-L(+)isoglutamine	F6C25H25N2O4	448.15	158-160	35.11
6	1-N-(4-aminobiphenyl)-2-N-(4-biphenylacetyl)-L(+)-isoglutamine	C31H29N2O4	410.16	164-166	17.00
7	1-N-(2-aminobenzoxazole)-2-N-(4-biphenylacetyl)-L(+)-isoglutamine	C26H24N3O5	430.16	144-146	39.70
8	1-N-(n-butyl)-2-N-(4-biphenylacetyl)-L(+)-isoglutamine	C23H29N2O4	475.12	140-142	21.60
9	1-N-(2-chlorobenzyl)-2-N-(4-biphenylacetyl)-L(+)-isoglutamine	ClC26H26N2O4	424.19	150-152	12.00

10	1-N-(n-pentyl)-2-N-(4-biphenylacetyl)-L(+)-isoglutamine	C24H31N2O4	566.51	170-172	31.51
11	1-N-(4-Nitrobenzyl)-2-N-(4-biphenylacetyl)-L(+)-isoglutamine	C26H26N3O6	431.14	154-156	20.00
12	1-N-(2-aminobenzimidazole)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine	C26H25N4O4	456.15	166-168	19.00
13	1-N-(orthophenylenediamine)-2-N-(4-biphenylacetyl)-L(+)-isoglutamine	C25H26N3O4	492.23	146-148	41.66
14	1-N-benzyl-2-N-(4-biphenylacetyl)-L(+)-isoglutamine	C26H27N2O4	457.14	142-144	44.77

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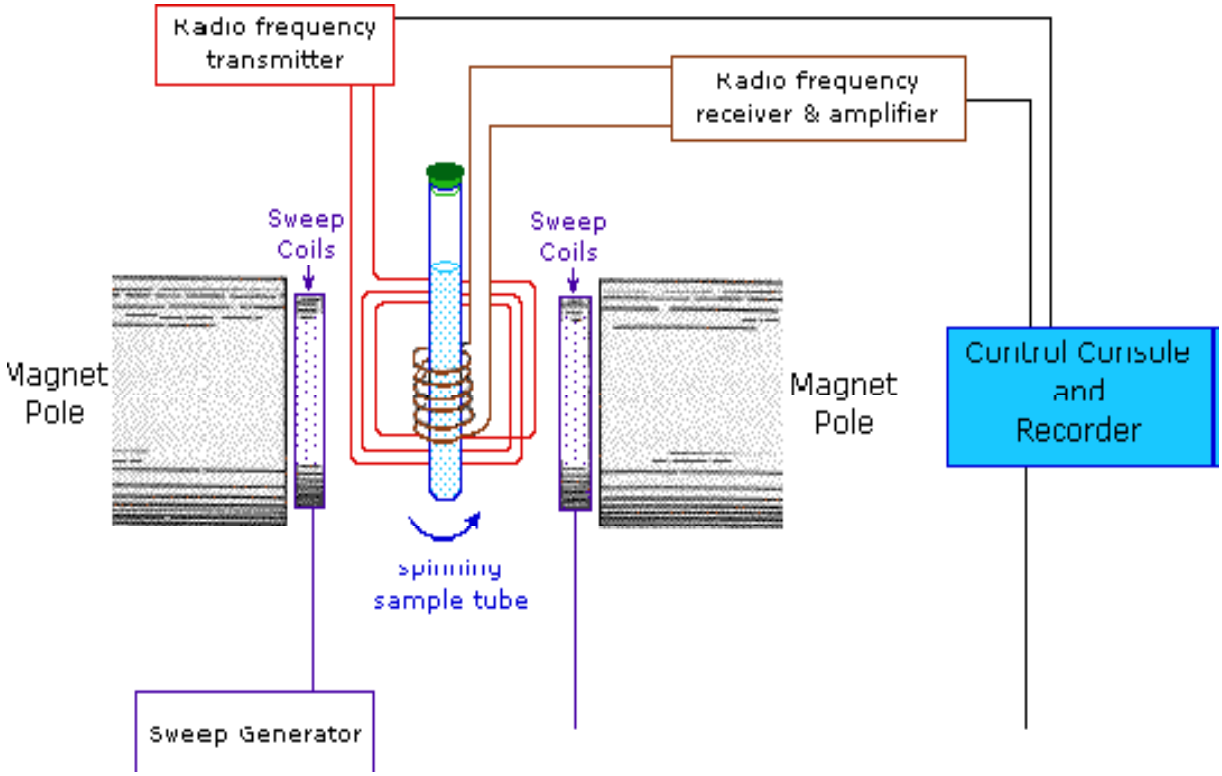
5. NMR ANALYSIS:

NMR spectroscopy means Nuclear magnetic resonance spectroscopy is a research technique that exploits the magnetic properties of certain atomic nuclei. It determines the physical and chemical properties of atoms or the carbon skeleton in the structure of molecules in which they are contained.

5.1. Basics Of Nuclear Magnetic Resonance Spectroscopy:

The nuclear magnetic resonance (NMR) spectroscopy experiment involves using energy in the form of electromagnetic radiation to pump the excess alpha oriented nuclei into the beta state. When the energy is removed, the energized nuclei relax back to the alpha state. The fluctuation of the magnetic field associated with this relaxation process is called resonance and this resonance can be detected and converted into the peaks we see in an NMR spectrum. In this context, it is relevant to note that different nuclear magnetic resonance spectrometers have different magnetic field strengths. Basic mechanism of NMR spectroscopy is shown in figure-1. For example, the NMR on the first floor of Park Hall has a relatively high field, superconducting magnet. Because the field is high (high enough to erase bank cards and interfere with pacemakers and watches), the frequency range needed to excite protons is relatively high. It is called a 300 MHz (MHz = megahertz, a hertz is a cycle per second - a frequency unit) spectrometer, referring to the excitation frequency. The NMR on the second floor of Park Hall has a much weaker electromagnet associated with it. It is a 60 MHz instrument. Since different NMRs have different operating frequencies, spectra cannot be compared from different machines if they are reported in frequency units. For this reason, the universal ppm (parts per million) units are used in NMR. Please note the following relationship between ppm and frequency.

$$\text{Chemical shift in ppm} = \frac{\text{peak position in Hz (relative to TMS)}}{\text{spectrometer frequency in MHz}}$$



Basic Mechanism of NMR spectroscopy

Now let us use these basic ideas to better understand and interpret NMR spectra.

1. Why do we see peaks? When the excited nuclei in the beta orientation start to relax back down to the alpha orientation, a fluctuating magnetic field is created. This fluctuating field generates a current in a receiver coil that is around the sample. The current is electronically converted into a peak. It is the relaxation that actually gives the peak not the excitation.

2. Why do we see peaks at different positions? Realize that in principle, a peak will be observed for every magnetically distinct nucleus in a molecule. This happens because nuclei that are not in identical structural situations do not experience the external magnetic field to the same extent. The nuclei are shielded or deshielded due to small local fields generated by circulating sigma, pi and lone pair electrons.

The Interpretation of Simple NMR Spectra

1. The number of peaks: The number of peaks is directly related to symmetry. If a compound has three significantly different types of hydrogens, it should have three different NMR absorptions.

2. The area under each absorption (the integral): The relative areas (or integrals) of the various absorptions in an NMR spectrum equals the relative number of hydrogens absorbing. If we know the molecular formula of a compound, we can use this ratio to figure out the actual number of each type of hydrogen. From the numbers of each type, we can infer the carbon structure. For example, with bromoethane, the relative areas under the NMR peaks are 2:3. This tells us that there is a group of two hydrogens that are the same and another group of three hydrogens that are the same. With your current knowledge of organic chemistry, it seems most likely that the compound has a methyl (-CH3) and a methylene (-CH2-) group. In other words, the most probable way to have three identical hydrogens is on a methyl group. The most probable way to have two identical hydrogens is in a methylene group.

Suppose you have a compound with the formula C5H12O and you are told that there are two NMR peaks, having the relative areas of 1:3. Can you come up with the structure of the compound?

3. The splitting pattern: For this semester, we will be using the n+1 rule as it applies to the simple structures we will be determining. You will see one more peak than the number of adjacent, but different hydrogens. Therefore, you can look at any peak and automatically know how many neighbors there are. This is crucial information because it allows you to start to hook atoms together in your structure. The problem is that people often confuse integral with splitting. So you must always remember this saying **"Integral tells you what is here and splitting tells you what is near"** This means that the integral tells you about the absorbing hydrogens and the splitting tells you about the neighbors. So what does it mean if you see a quintet with an area of two in a spectrum?

4. The position of the peak or the chemical shift (δ): This tells you about the electronic environment (the electronic environment directly relates to the magnetic environment) of the absorbing hydrogens. It will tell you if there are pi bonds or electronegative atoms nearby, etc. There are nice tables available that organize how different groups effect the frequency of

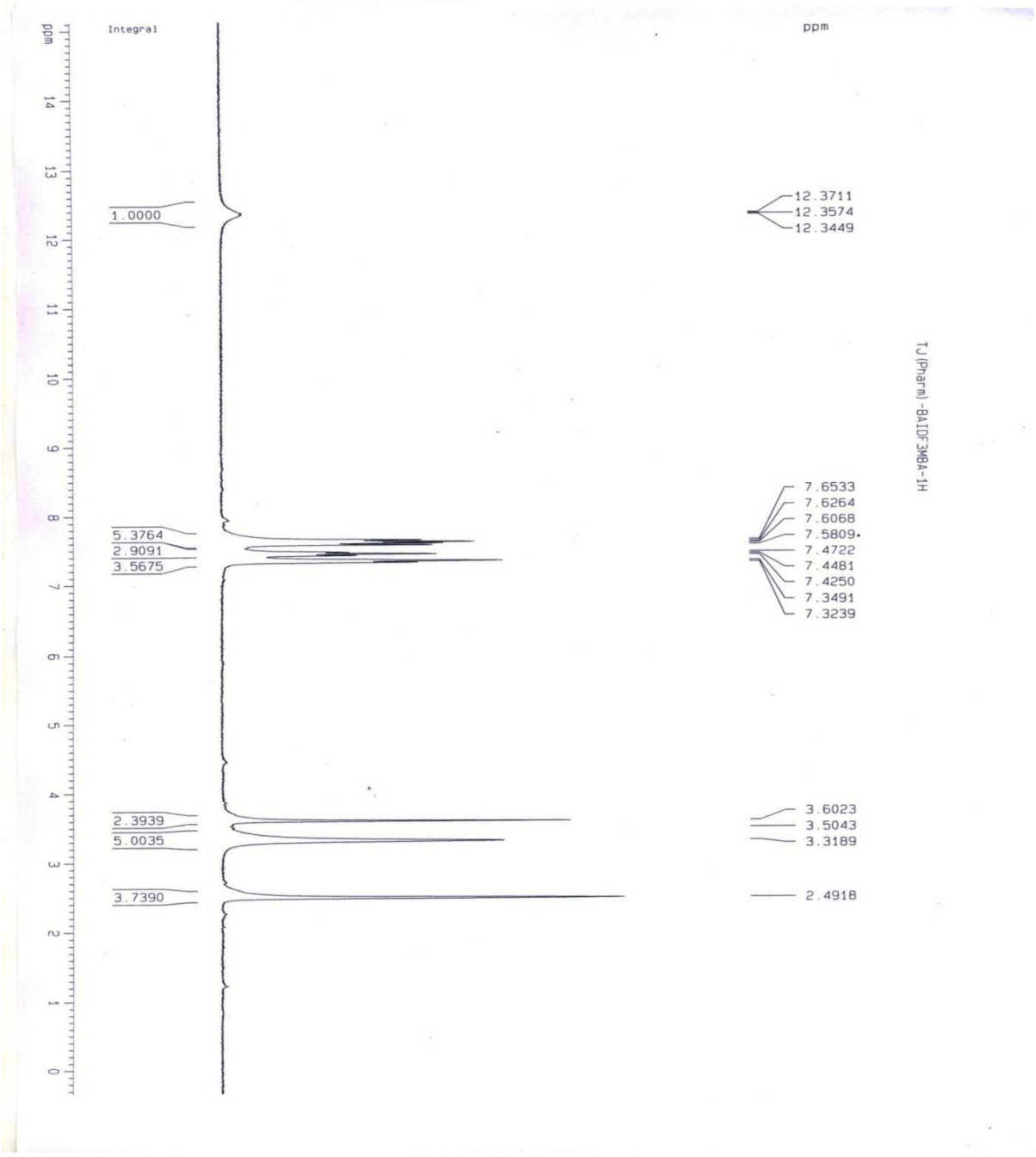
absorptions and in lab you will always have these tables available to you. Yes, you will even have them on exams. A good rule of thumb when you are solving spectra is that the closer a hydrogen is to an electronegative atom the higher the ppm position. This little rule only works if the hydrogen is two or more bonds away from the atom. You will soon see the utility of this when you begin your problems in the workshop. It is also useful to keep in your head that aromatic hydrogens absorb at around 7 ppm.

5.2. NMR Spectroscopic Analysis of Synthesized Compounds:

NMR Spectroscopic Analysis Of Synthesized Compounds

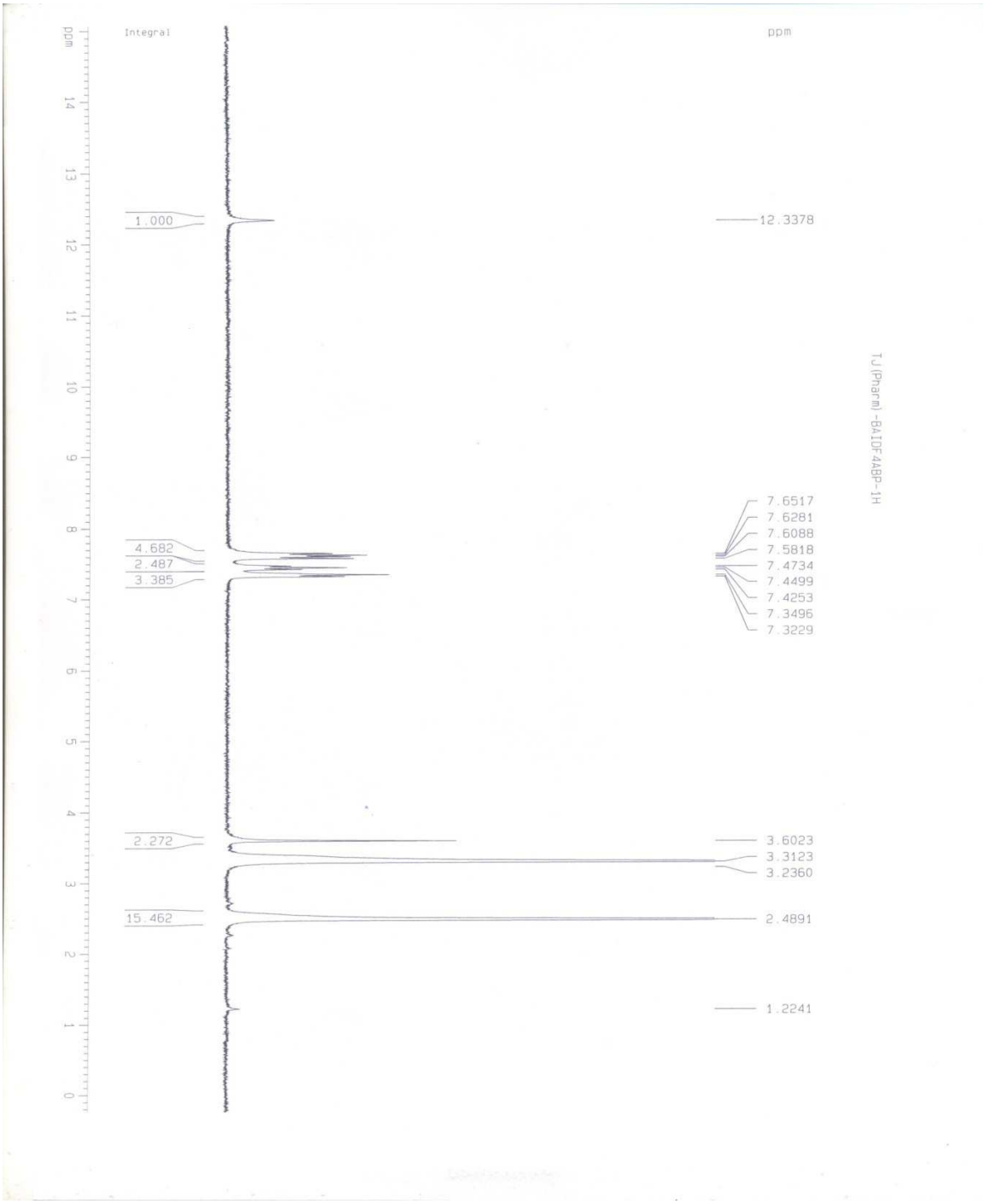
(A)1-N-3,5,-Bisdi(trifluoromethyl)benzyl-2-N-(4-biphenylacetyl)-L(+)isoglutamine

1HNMR (300 MHz, DMSO-D6): δ 2.49 (m, 1H amine), δ 3.31 (m, 2H, ali-CH), δ 7.32-7.65 (m, 12H, biphenyl CH), δ 12.37 (m, carboxylic acid).

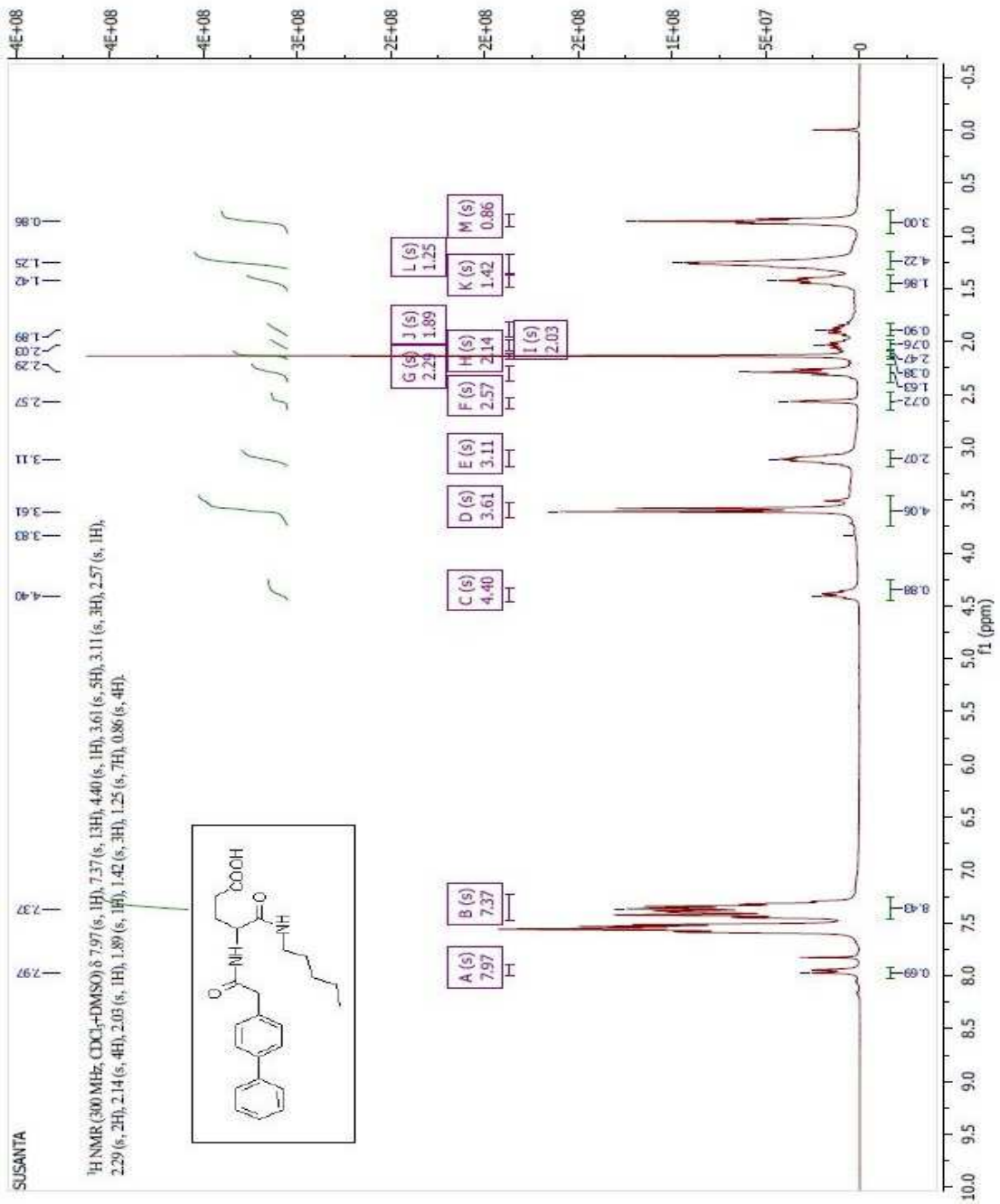


(B)1-N-(4-aminobiphenyl)-2-N-(4-biphenylacetyl)-L(+)-isoglutamine

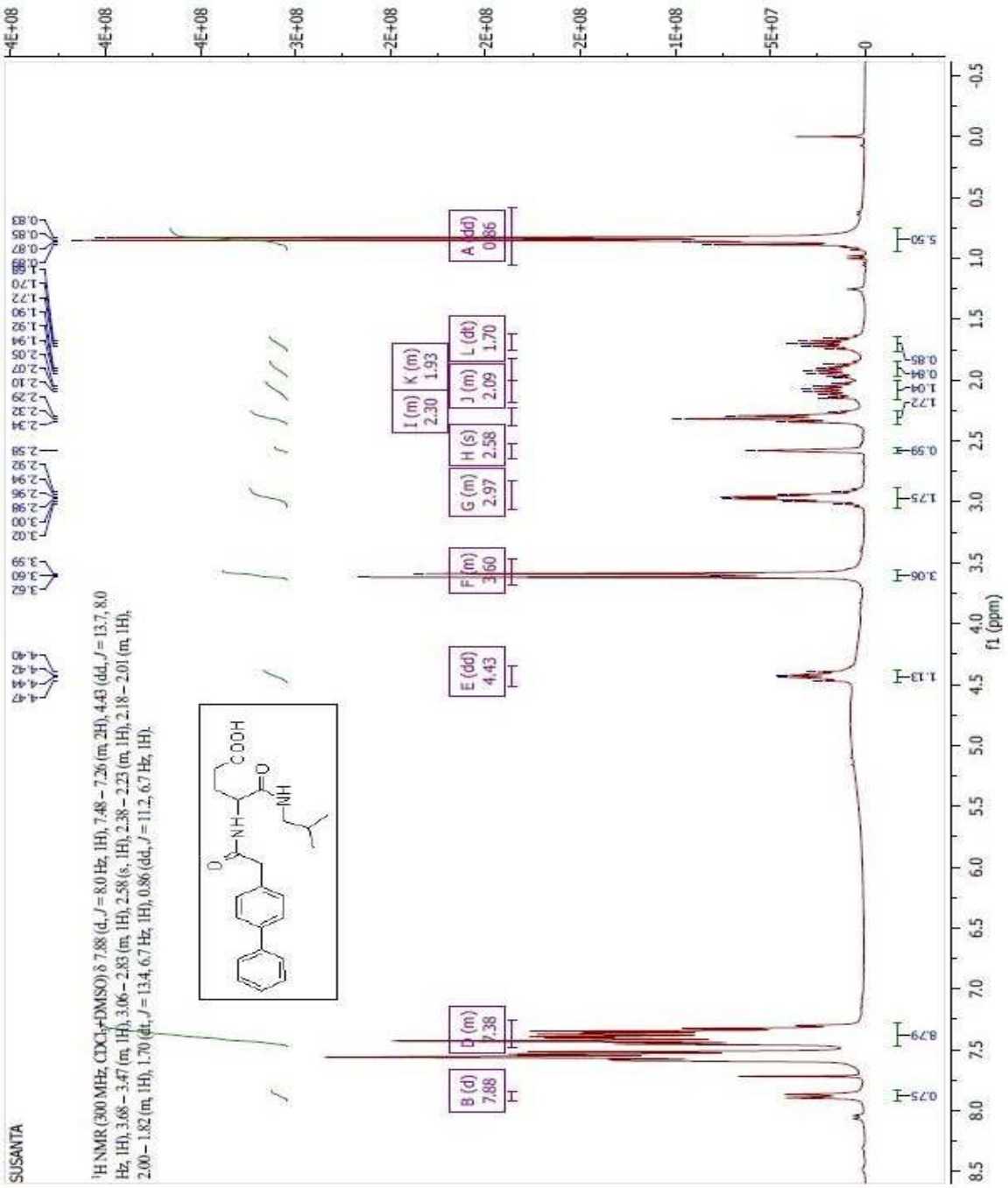
1HNMR (300 MHz, DMSO-D6): δ1.22(m, Ar.NH2), δ 2.48 (m, 1H amine), δ 3.31 (m, 2H, ali-CH), δ 7.32-7.65 (m, 12H, biphenyl CH), δ 12.37 (m, carboxylic acid).



(C)1-N-(n-pentyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine

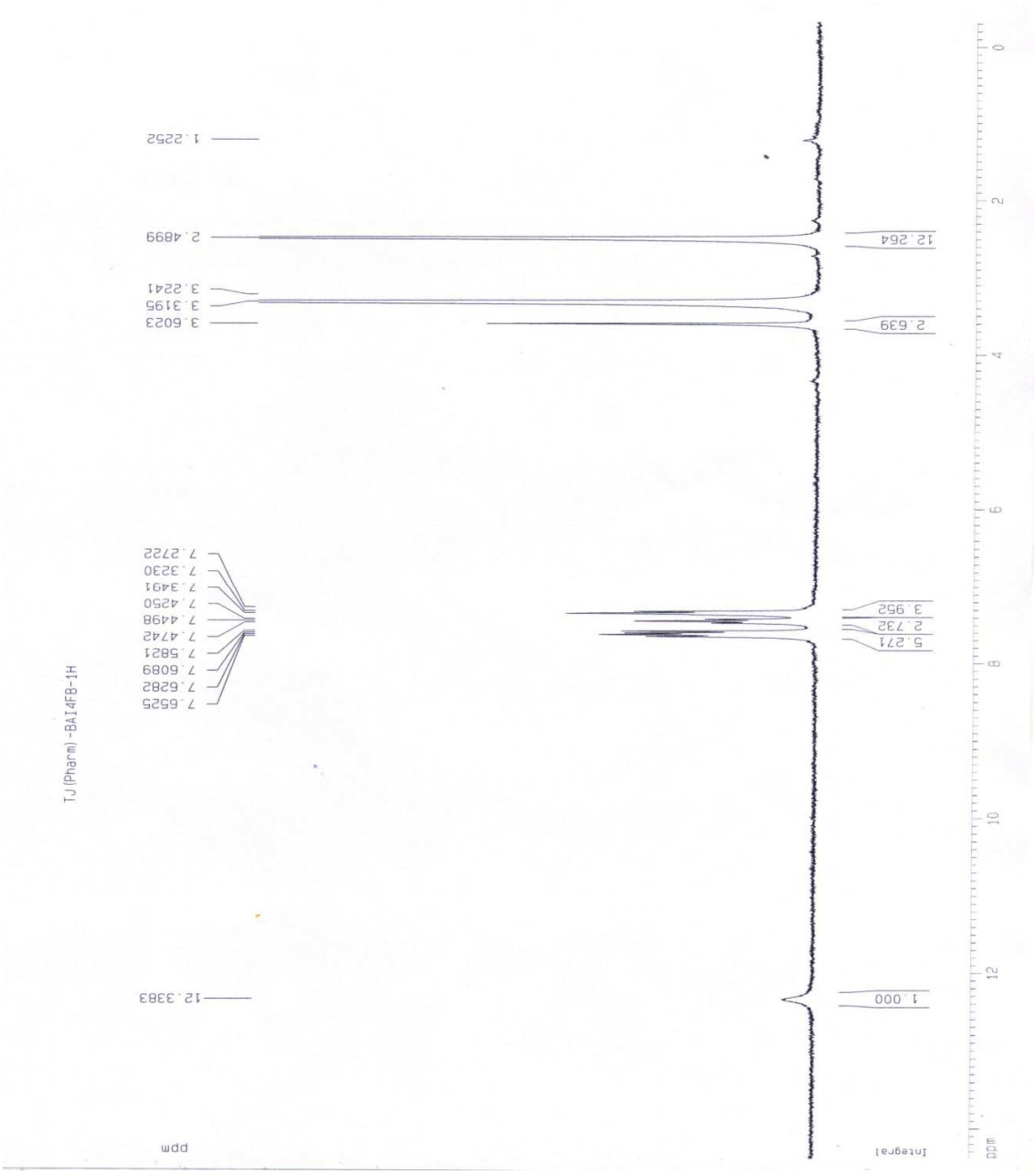


(D)1-N-isobutyl-2-N-(4-biphenylacetyl)-L (+)-isoglutamine



(E) 1-N-(4-Fluorobenzyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine

1HNMR (300 MHz, DMSO-D6): δ1.22(m, Ar.NH2), δ 2.48 (m, 1H amine), δ 3.31 (m, 2H, ali-CH), δ7.27-7.65 (m, 12H, biphenyl CH), δ 12.33 (m, carboxylic acid).



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6. FT-IR (Fourier Transform Infrared) SPECTROSCOPIC ANALYSIS:

Infrared spectroscopy is the spectroscopy that deals with the infrared region of the electromagnetic spectrum, that is light with a longer wavelength and lower frequency than visible light. It covers a range of techniques and is mostly based on absorption spectroscopy. A common laboratory instrument that uses this technique is a Fourier transform infrared (FTIR) spectrometer. It can be used for:

- Identifying an unknown compound by matching its spectra to the spectra of known standard compound.
- To monitor chemical reactions in-situ.
- Structural determination of the compounds.

The FT-IR spectra of the synthesised compounds were recorded at Perkin Elmer Spectrum 100 FTIR spectrophotometer. Running the spectrum of 0.05mm polystyrene film did the finer calibration of the machine. The frequencies were expressed in cm-1.

6.1. Number of vibrational modes:

In order for a vibrational mode in a molecule to be "IR active", it must be associated with changes in the dipole. A permanent dipole is not necessary, as the rule requires only a change in dipole moment.

A molecule can vibrate in many ways, and each way is called a vibrational mode. For molecules with N number of atoms in them, linear molecules have 3N – 5 degrees of vibrational modes, whereas nonlinear molecules have 3N – 6 degrees of vibrational modes (also called vibrational degrees of freedom). As an example H2O, a non-linear molecule, will have $3 \times 3 – 6 = 3$ degrees of vibrational freedom, or modes.

Simple diatomic molecules have only one bond and only one vibrational band. If the molecule is symmetrical, e.g. N2, the band is not observed in the IR spectrum, but only in the Raman spectrum. Asymmetrical diatomic molecules, e.g. CO, absorb in the IR spectrum. More complex molecules have many bonds, and their vibrational spectra are correspondingly more complex, i.e. big molecules have many peaks in their IR spectra.

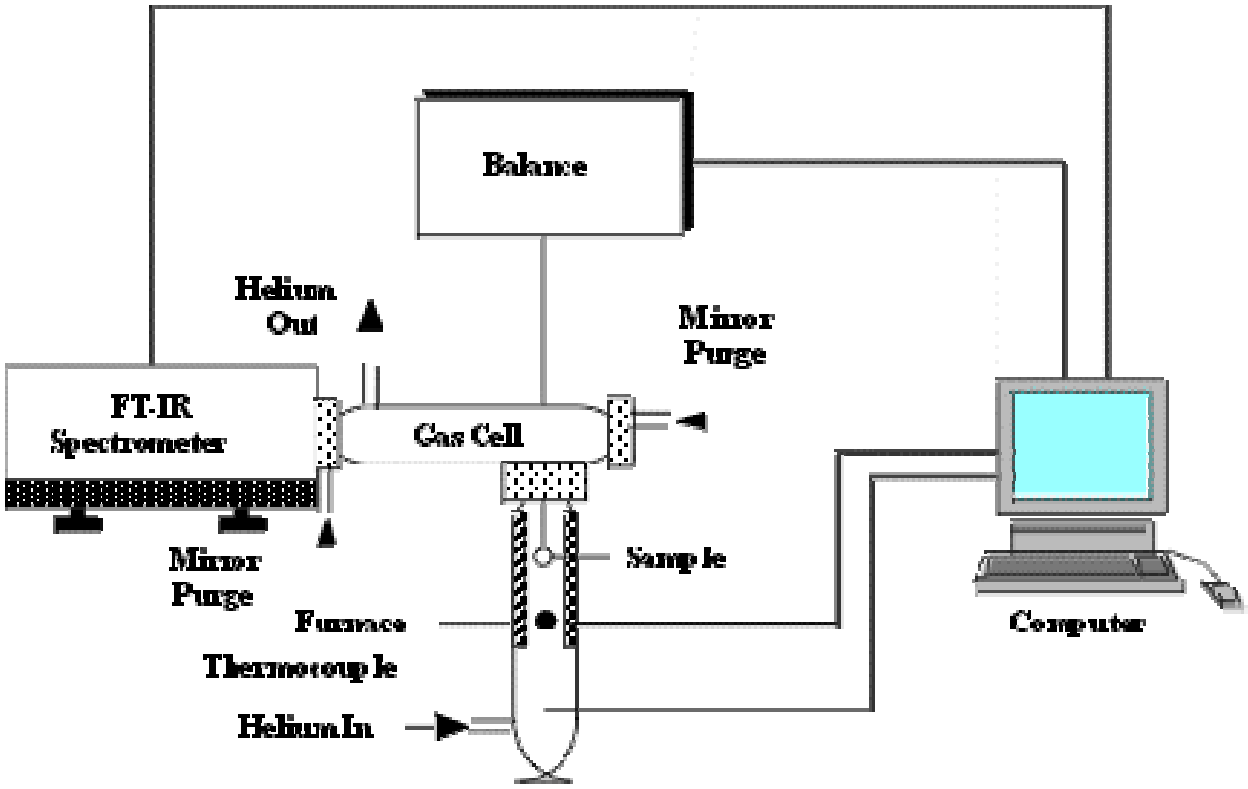
The atoms in a CH2X2 group, commonly found in organic compounds and where X can

represent any other atom, can vibrate in nine different ways. Six of these involve only theCH2 portion: **symmetric and anti-symmetric stretching, scissoring, rocking, wagging** and **twisting**. Note, that because CH2 is attached to X2 it has 6 modes, unlike H2O, which only has 3 modes. The rocking, wagging, and twisting modes do not exist for H2O, since they are rigid body translations and no relative displacements exist.

6.2. IR Spectroscpic Proceedure:

The infrared spectrum of a sample is recorded by passing a beam of infrared light through the sample. When the frequency of the IR is the same as the vibrational frequency of a bond, absorption occurs. Examination of the transmitted light reveals how much energy was absorbed at each frequency (or wavelength). This can be achieved by scanning the wavelength range using a monochromator. Alternatively, the whole wavelength range is measured at once using a Fourier transform instrument and then a transmittance or absorbance spectrum is generated using a dedicated procedure. Analysis of the position, shape and intensity of peaks in this spectrum reveals details about the molecular structure of the sample.

This technique works almost exclusively on samples with covalent bonds. Simple spectra are obtained from samples with few IR active bonds and high levels of purity. More complex molecular structures lead to more absorption bands and more complex spectra. The technique has been used for the characterization of very complex mixtures. Spectra issues with infrared fluorescence are rare. The IR analytic pathway is shown below-



FTIR analytical pathway

6.2.a. Sample Preparation:

Gaseous samples require a sample cell with a long pathlength to compensate for the diluteness. The pathlength of the sample cell depends on the concentration of the compound of interest. A simple glass tube with length of 5 to 10 cm equipped with infrared-transparent windows at the both ends of the tube can be used for concentrations down to several hundred ppm. Sample gas concentrations well below ppm can be measured with a White's cell in which the infrared light is guided with mirrors to travel through the gas. White's cells are available with optical pathlength starting from 0.5 m up to hundred meters.

Liquid samples can be sandwiched between two plates of a salt (commonly sodium chloride, or common salt, although a number of other salts such as potassium bromide or calcium fluoride are also used). The plates are transparent to the infrared light and do not introduce any lines onto the spectra.

Solid samples can be prepared in a variety of ways. One common method is to crush the sample with an oily mulling agent (usually Nujol) in a marble or agate mortar, with a pestle. A thin film of the mull is smeared onto salt plates and measured. The second method is to grind a quantity of the sample with a specially purified salt (usually potassium bromide) finely (to remove scattering effects from large crystals). This powder mixture is then pressed in a mechanical press to form a translucent pellet through which the beam of the spectrometer can pass. A third technique is the "cast film" technique, which is used mainly for polymeric materials. The sample is first dissolved in a suitable, non hygroscopic solvent. A drop of this solution is deposited on surface of KBr or NaCl cell. The solution is then evaporated to dryness and the film formed on the cell is analysed directly. Care is important to ensure that the film is not too thick otherwise light cannot pass through. This technique is suitable for qualitative analysis. The final method is to use microtomy to cut a thin (20–100 μm) film from a solid sample. This is one of the most important ways of analysing failed plastic products for example because the integrity of the solid is preserved.

In photoacoustic spectroscopy the need for sample treatment is minimal. The sample, liquid or solid, is placed into the sample cup which is inserted into the photoacoustic cell which is then sealed for the measurement. The sample may be one solid piece, powder or basically in any form for the measurement. For example, a piece of rock can be inserted into the sample cup and the spectrum measured from it.

It is important to note that spectra obtained from different sample preparation methods will look slightly different from each other due to differences in the samples' physical states.

6.2.b. Compare to Reference:

To take the infrared spectrum of a sample, it is necessary to measure both the sample and a "reference" (or "control"). This is because each measurement is affected by not only the light-absorption properties of the sample, but also the properties of the instrument (for example, what light source is used, what infrared detector is used, etc.). The reference measurement makes it possible to eliminate the instrument influence. Mathematically, the sample transmission spectrum is divided by the reference transmission spectrum.

The appropriate "reference" depends on the measurement and its goal. The simplest reference measurement is to simply remove the sample (replacing it by air). However, sometimes a different reference is more useful. For example, if the sample is a dilute solute dissolved in water

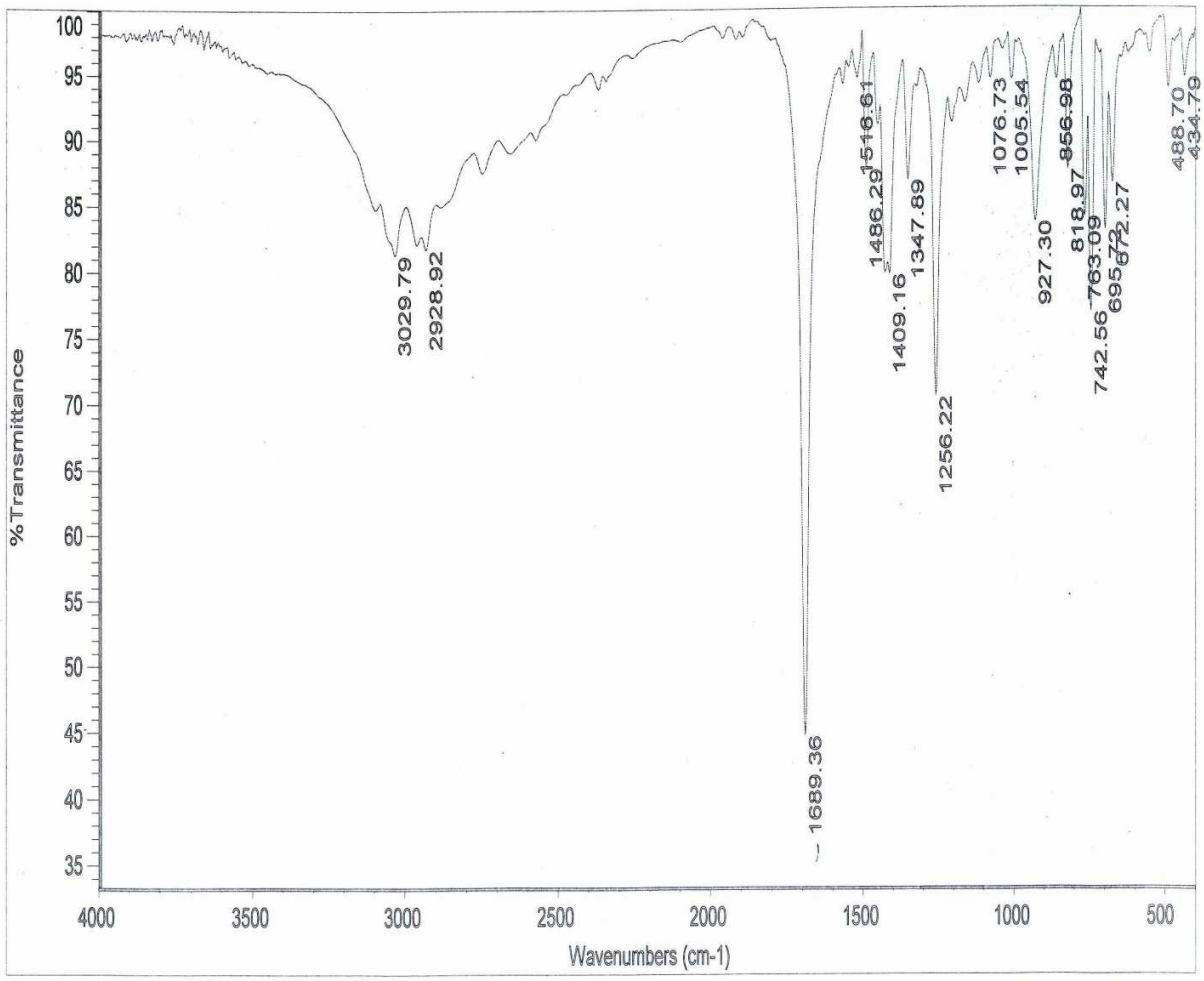
in a beaker, then a good reference measurement might be to measure pure water in the same beaker. Then the reference measurement would cancel out not only all the instrumental properties (like what light source is used), but also the light-absorbing and light-reflecting properties of the water and beaker, and the final result would just show the properties of the solute (at least approximately).

A common way to compare to a reference is sequentially: first measure the reference, then replace the reference by the sample and measure the sample. This technique is not perfectly reliable; if the infrared lamp is a bit brighter during the reference measurement, then a bit dimmer during the sample measurement, the measurement will be distorted. More elaborate methods, such as a "two-beam" setup (see figure), can correct for these types of effects to give very accurate results. The Standard addition method can be used to statistically cancel these errors.

6.3. FT-IR ANALYSIS OF SYNTHESIZED COMPOUNDS

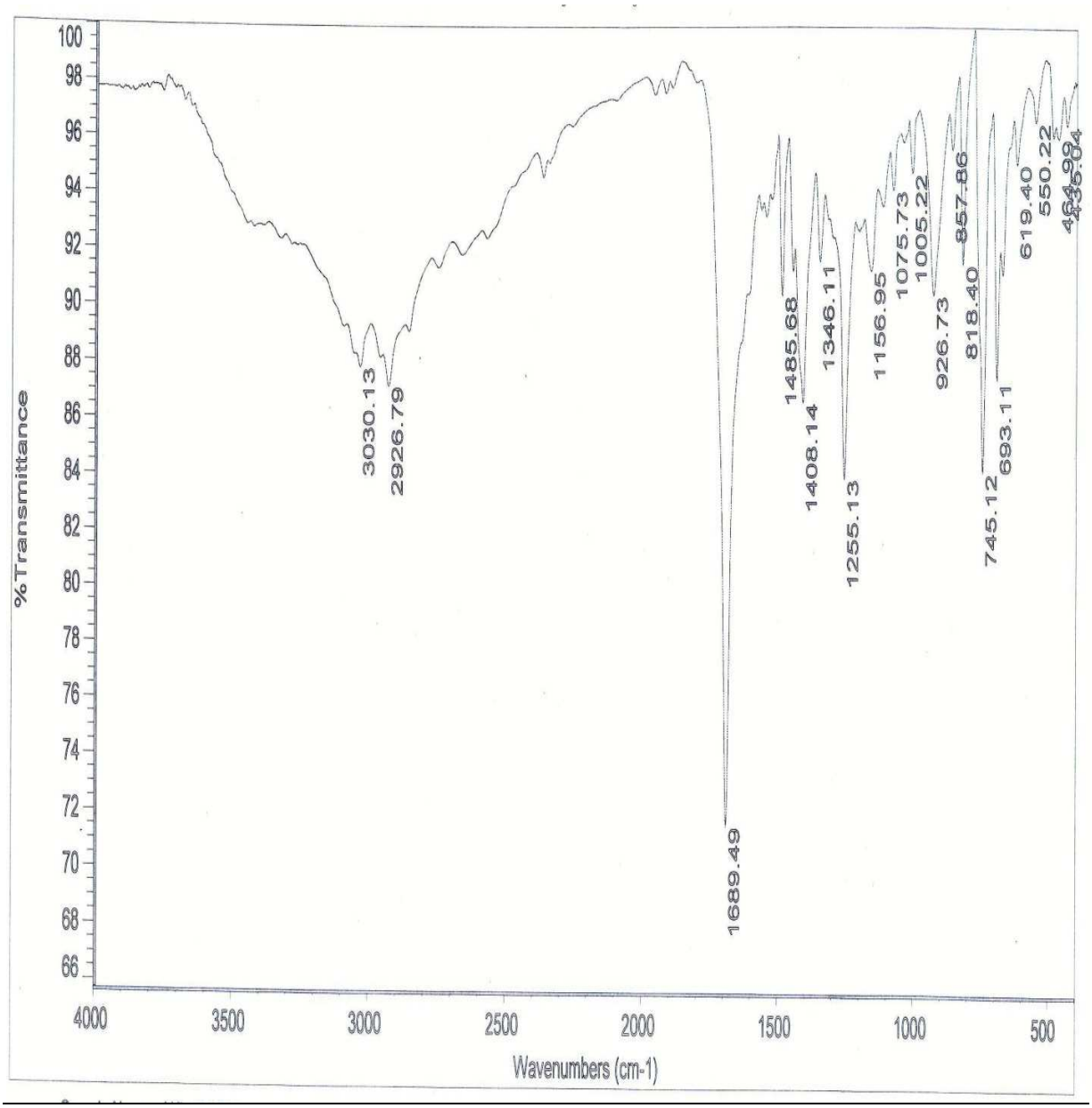
1-N-(4-Fluorobenzyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine

IR (KBr, cm-1): 3029 (N-H str of CONH),2928 (=C-H str. In Ar.), 1689 (C=O str. biphenyl ring), 1518,1486 (C=C bend of Ar), 1409 (in plane OH bending), 1347 (C-H bend),1256 (F-aryl), 1076 (carbon ring in cyclic comps.), 1000-500 (Ar. C-H bend), 488 (-C-C=O bend).



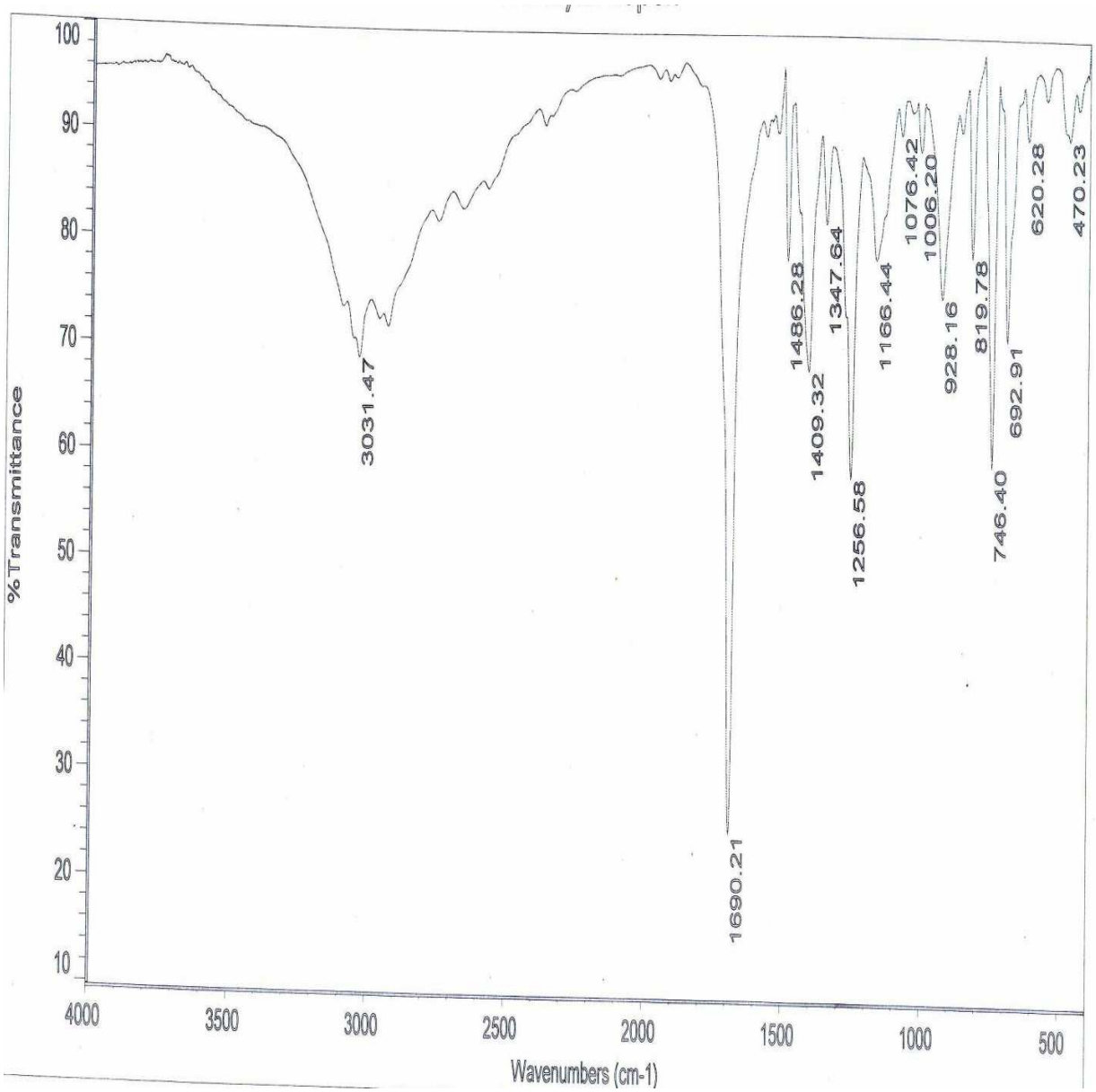
1-N-phenyl-2-N-(4-biphenylacetyl)-L (+)-isoglutamine

IR (KBr, cm-1): 3030 (N-H str of CONH),2926 (=C-H str. In Ar.), 1689 (C=O str. biphenyl ring),1485 (C=C bend of Ar), 1408 (in plane OH bending), 1346 (C-H bend),1255 (N-aryl), 1005 (carbon ring in cyclic comps.), 1000-500 (Ar. C-H bend), 488 (-C-C=O bend).



1-N-3,5,-Bisdi(trifluoromethyl)benzyl-2-N-(4-biphenylacetyl)-L(+)isoglutamine

IR (KBr, cm-1): 3031 (N-H str of CONH), 1690 (C=O str. biphenyl ring),1486 (C=C bend of Ar), 1409 (in plane OH bending), 1347 (C-H bend), 1006 (carbon ring in cyclic comps.), 1000-500 (Ar. C-H bend),620 (Ar-CF_3 str.), 488 (-C-C=O bend).



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7. MASS SPECTROSCOPY ANALYSIS:

This technique basically studies the effect of ionizing energy on molecules. It depends upon chemical reactions in the gas phase in which sample molecules are consumed during the formation of ionic and neutral species.

Mass spectrometry is a powerful analytical technique used to quantify known materials, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances.

7.1. Basic principle:

A mass spectrometer generates multiple ions from the sample under investigation, it then separates them according to their specific mass-to-charge ratio (m/z), and then records the relative abundance of each ion type.

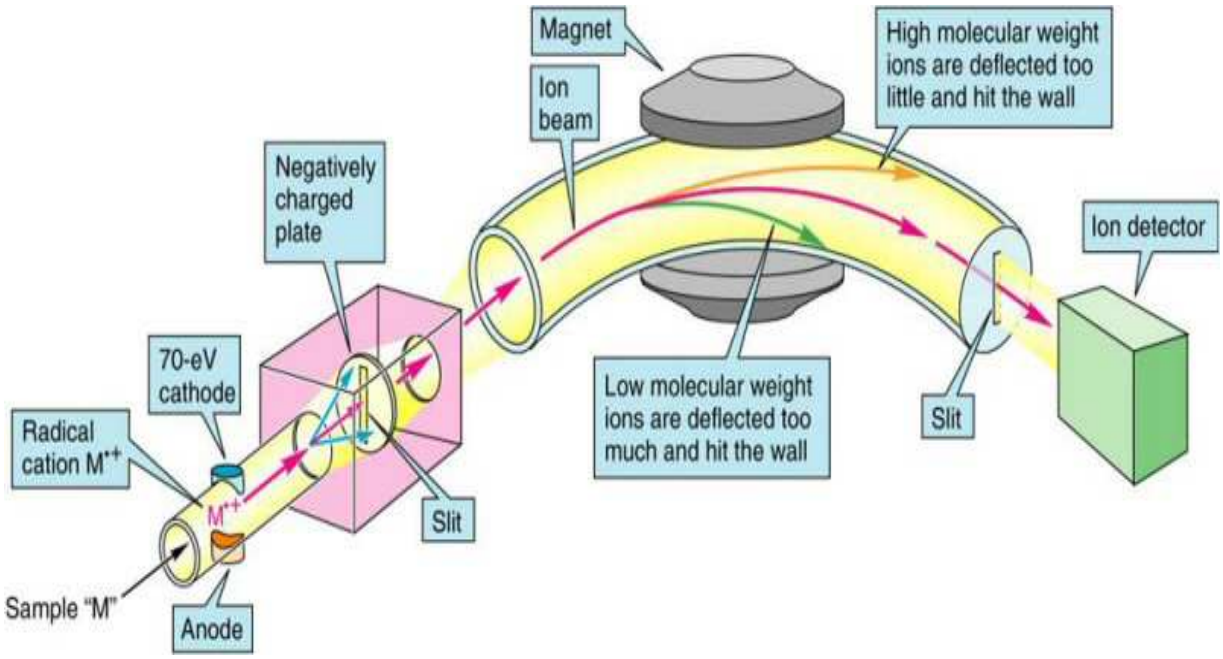
The first step in the mass spectrometric analysis of compounds is the production of gas phase ions of the compound, basically by electron ionization. This molecular ion undergoes fragmentation. Each primary product ion derived from the molecular ion, in turn, undergoes fragmentation, and so on. The ions are separated in the mass spectrometer according to their mass-to-charge ratio, and are detected in proportion to their abundance. A mass spectrum of the molecule is thus produced. It displays the result in the form of a plot of ion abundance versus mass-to-charge ratio. Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound.

7.2. Components:

The instrument consists of three major components:

1. Ion Source: For producing gaseous ions from the substance being studied.
2. Analyzer: For resolving the ions into their characteristics mass components according to their mass-to-charge ratio.

3. Detector System: For detecting the ions and recording the relative abundance of each resolved ionic species. In addition, a sample introduction system is necessary to admit the samples to be studied to the ion source while maintaining the high vacuum requirements (~10-6 to 10-8 mm of mercury) of the technique; and a computer is required to control the instrument, acquire and manipulate data, and compare spectra to reference libraries. The components of Mass Spectrometer is shown below



Components of a Mass Spectroscopy

With all the above components, a mass spectrometer should always perform the following processes:

1.Produce ions from the sample in the ionization source.

2. Separate these ions according to their mass-to-charge ratio in the mass analyzer.

3. Eventually, fragment the selected ions and analyze the fragments in a second analyzer.

4. Detect the ions emerging from the last analyzer and measure their abundance with the detector that converts the ions into electrical signals.

5. Process the signals from the detector that are transmitted to the computer and control the instrument using feedback.

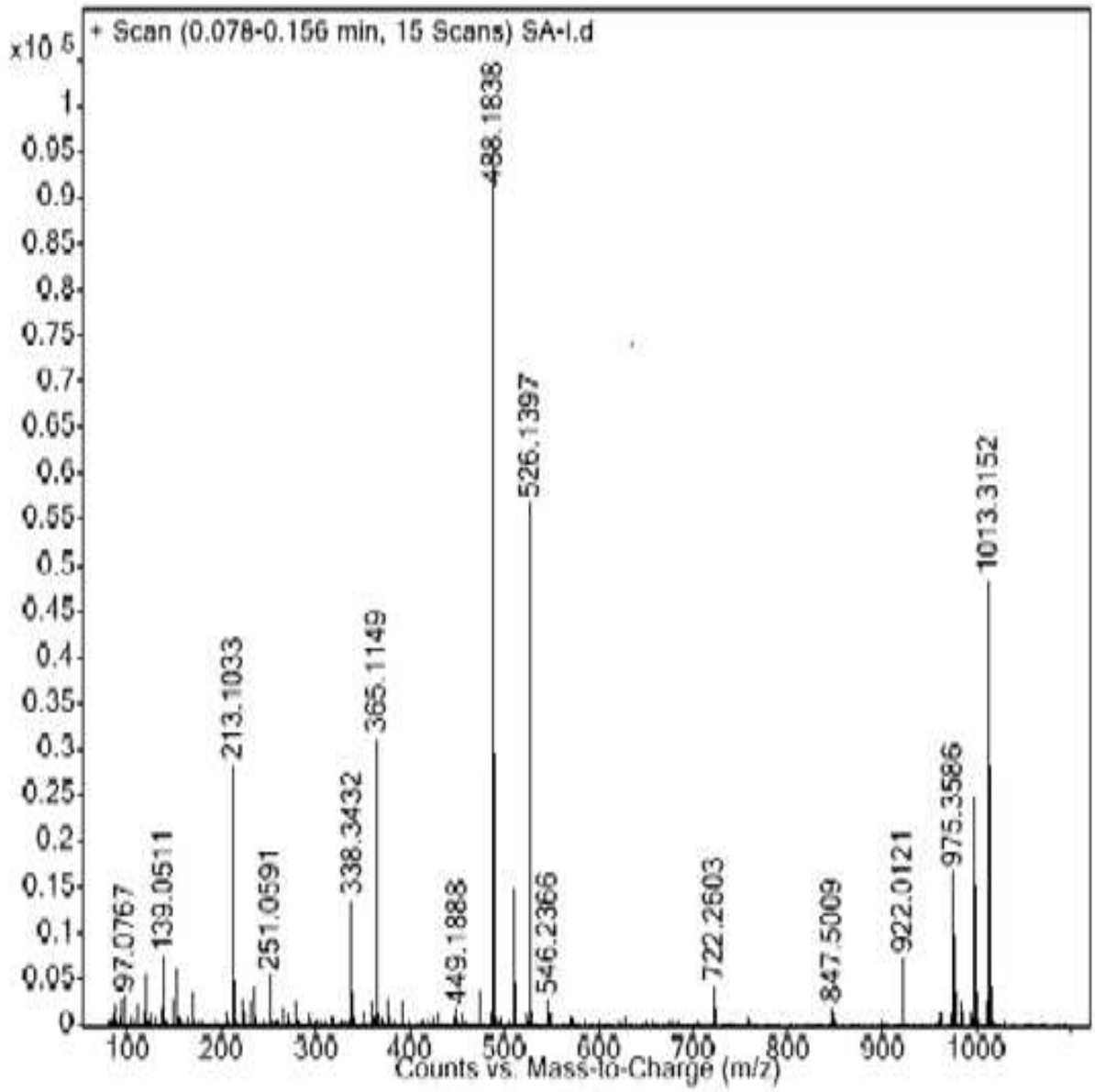
7.3. MASS SPECTROMETRIC ANALYSIS OF SYNTHESIZED COMPOUNDS:

1-N-(4-Fluorobenzyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine

Molecular weight- 488

M/z(M+H)=325

AnalysisFileName	AcquiredTime	InstrumentName
SA-I.d	2-Mar-2016	LC-QTOF MS/MS

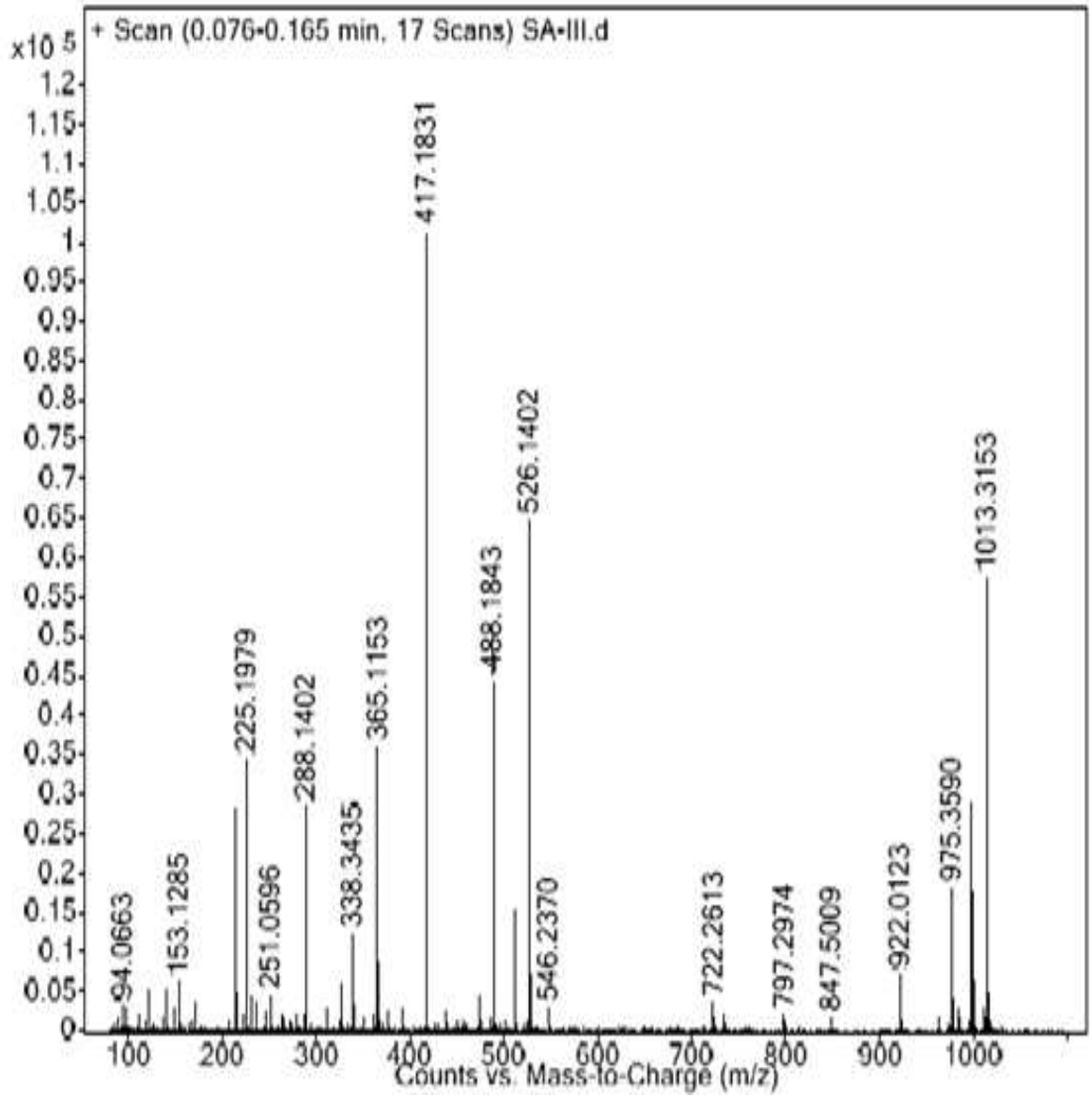


1-N-phenyl-2-N-(4-biphenylacetyl)-L (+)-isoglutamine

Molecular weight- 417

M/z(M+H)=325

AnalysisFileName	AcquiredTime	InstrumentName
SA-III.d	2-Mar-2016	LC-QTOF MS/MS

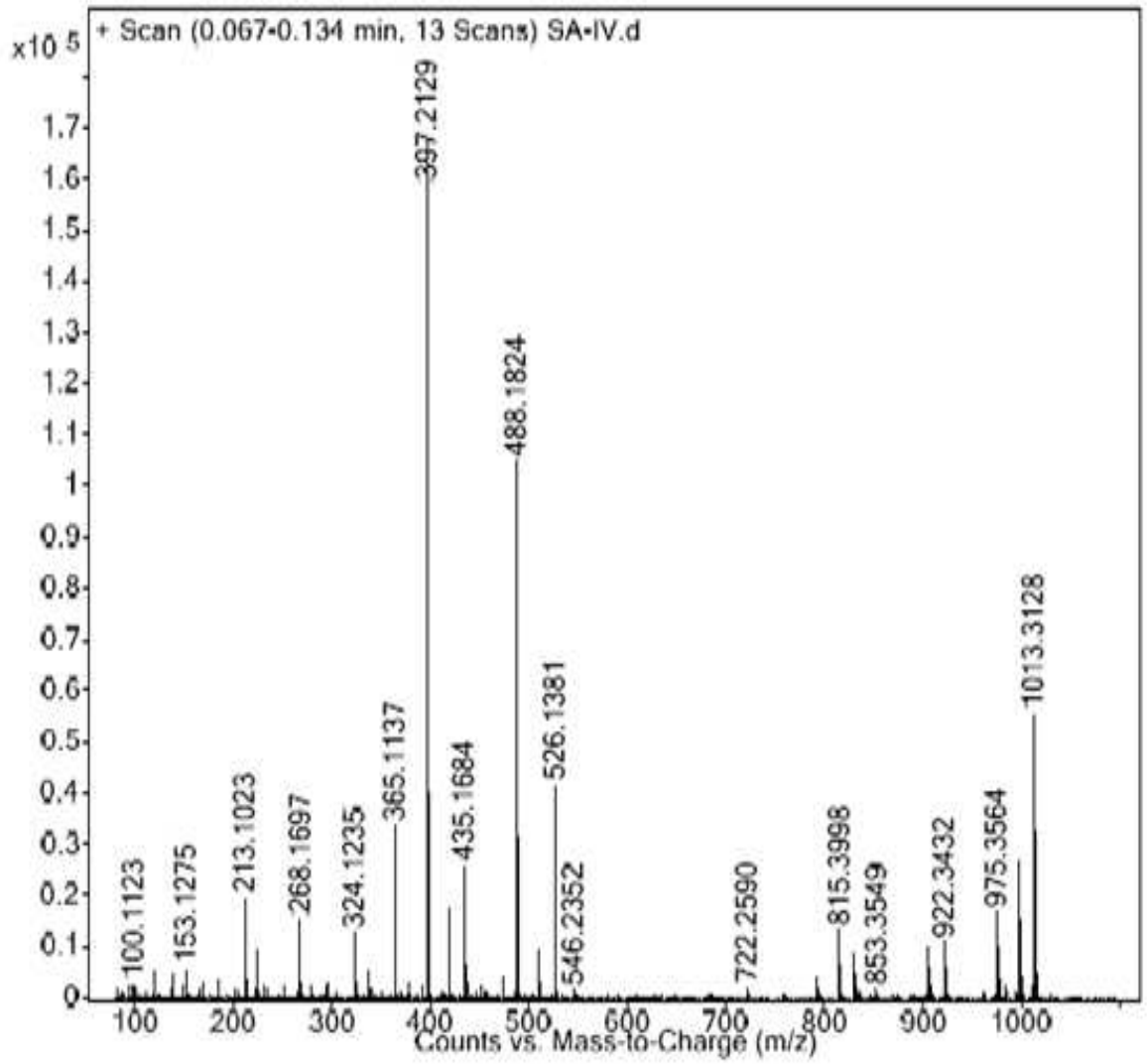


1-N-isobutyl-2-N-(4-biphenylacetyl)-L (+)-isoglutamine

Molecular weight- 397

M/z(M+H)=325

AnalysisFileName	AcquiredTime	InstrumentName
SA-IV.d	2-Mar-2016	LC-QTOF MS/MS

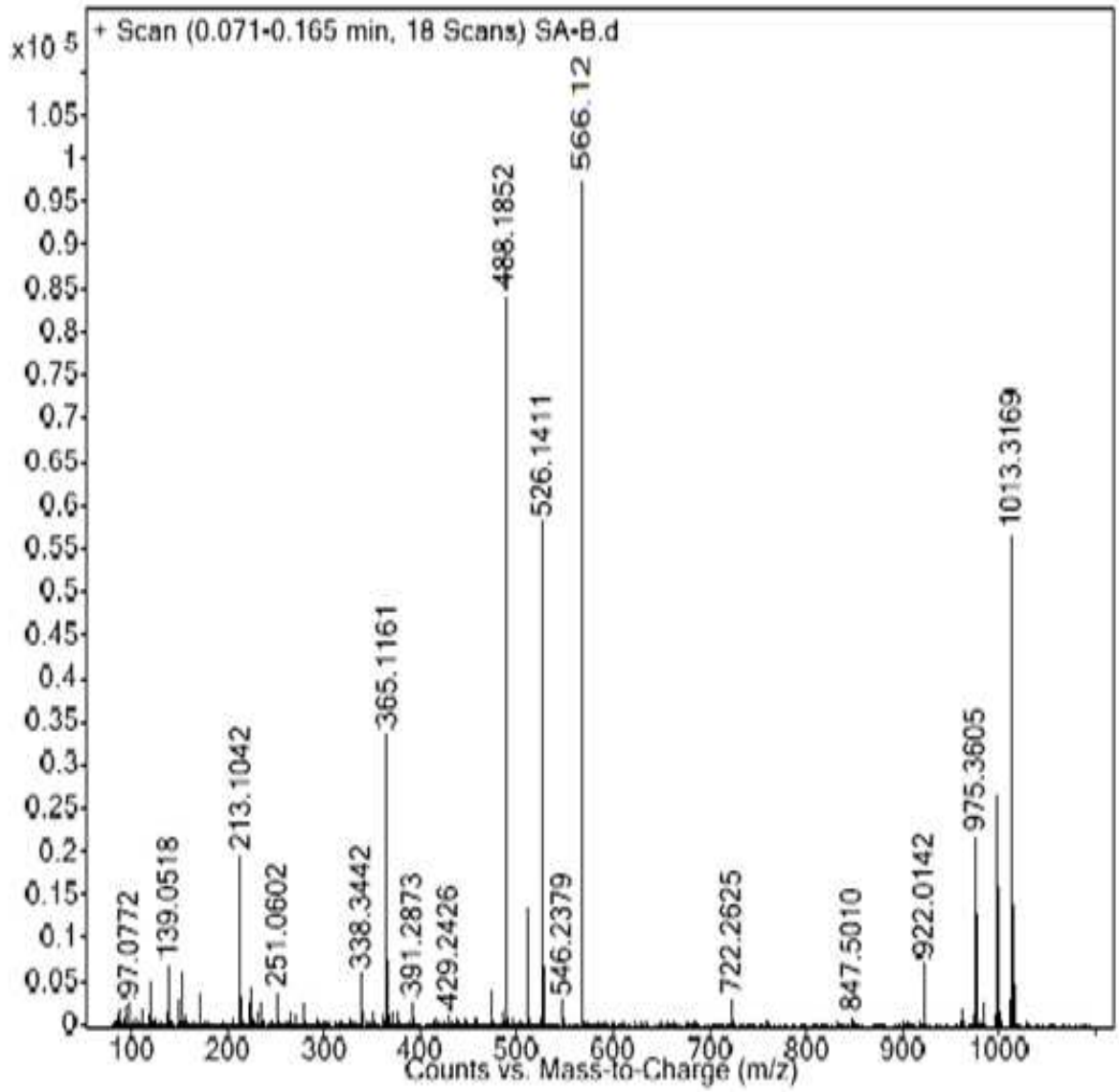


1-N-3,5,-Bisdi(trifluoromethyl)benzyl-2-N-(4-biphenylacetyl)-L(+)isoglutamine

Molecular weight- 566

M/z(M+H)=325

AnalysisFileName	AcquiredTime	InstrumentName
SA-B.d	2-Mar-2016	LC-QTOF MS/MS

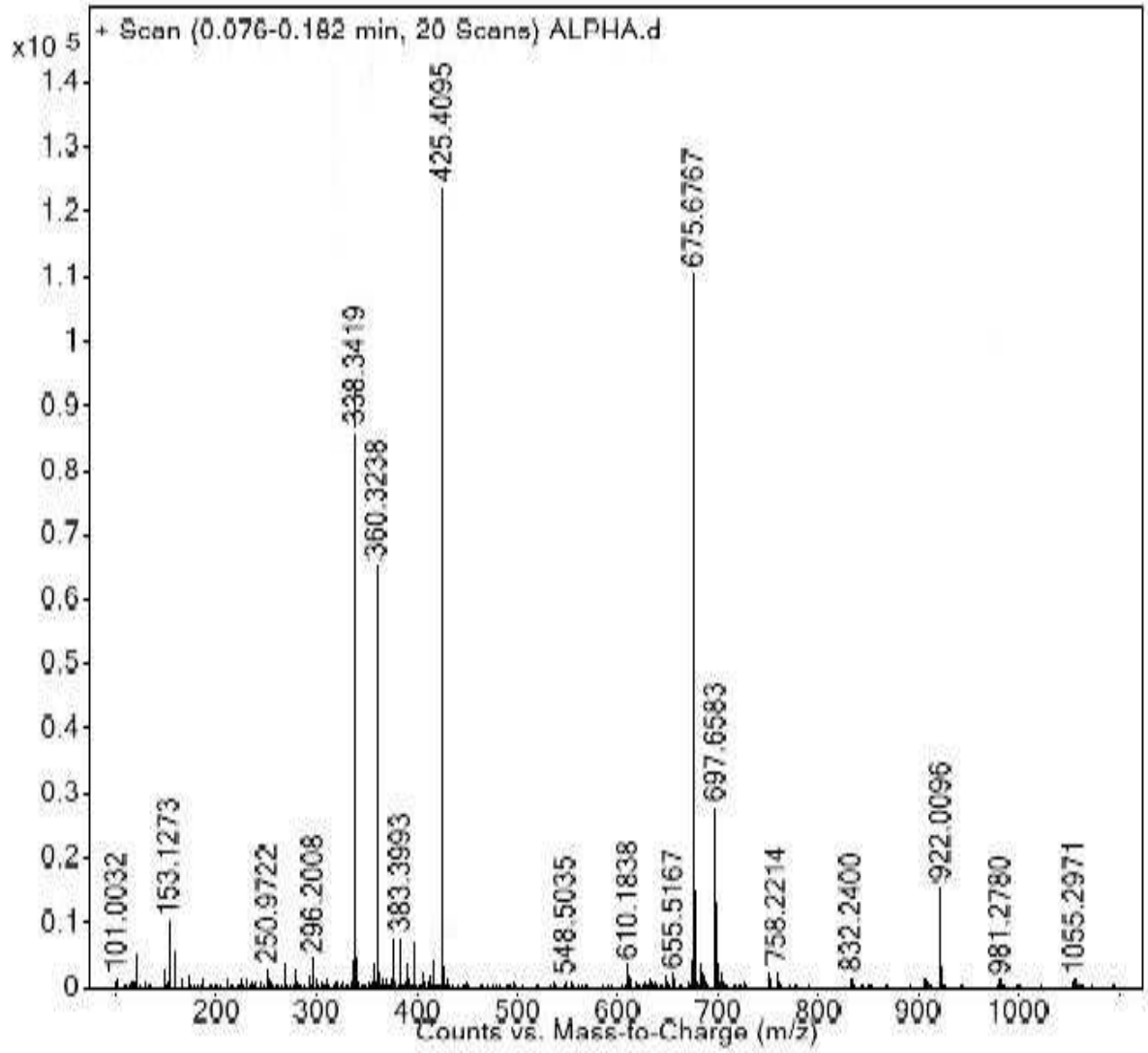


1-N-(n-hexyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine

Molecular weight- 425

M/z(M+H)=325

AnalysisFileName	AcquiredTime	InstrumentName
ALPHA.d	22-Apr-2016	LC-QTOF MS/MS

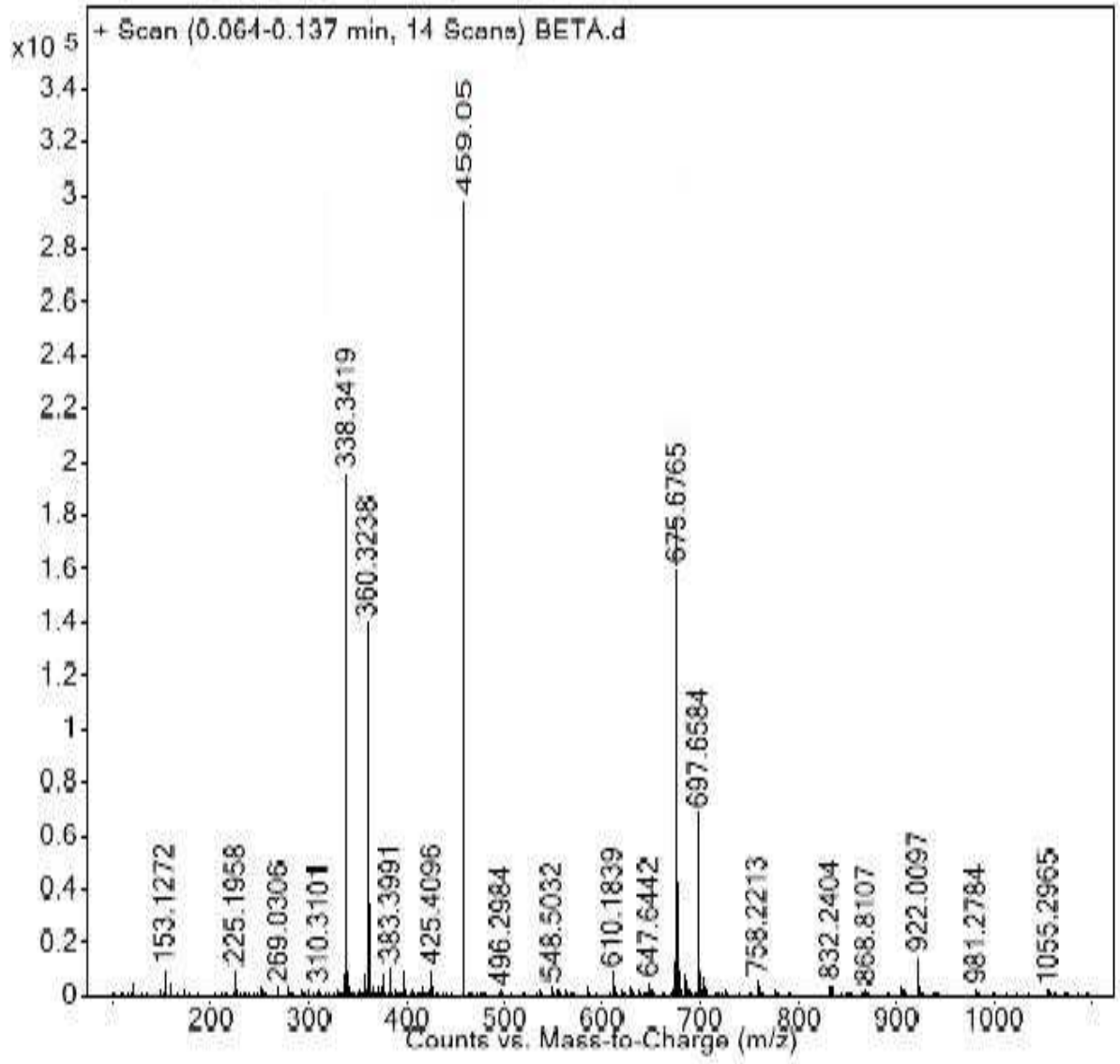


1-N-(2-aminobenzoxazole)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine

Molecular weight- 459

M/z(M+H)=325

AnalysisFileName	AcquiredTime	InstrumentName
BETA.d	22-Apr-2016	LC-QTOF MS/MS

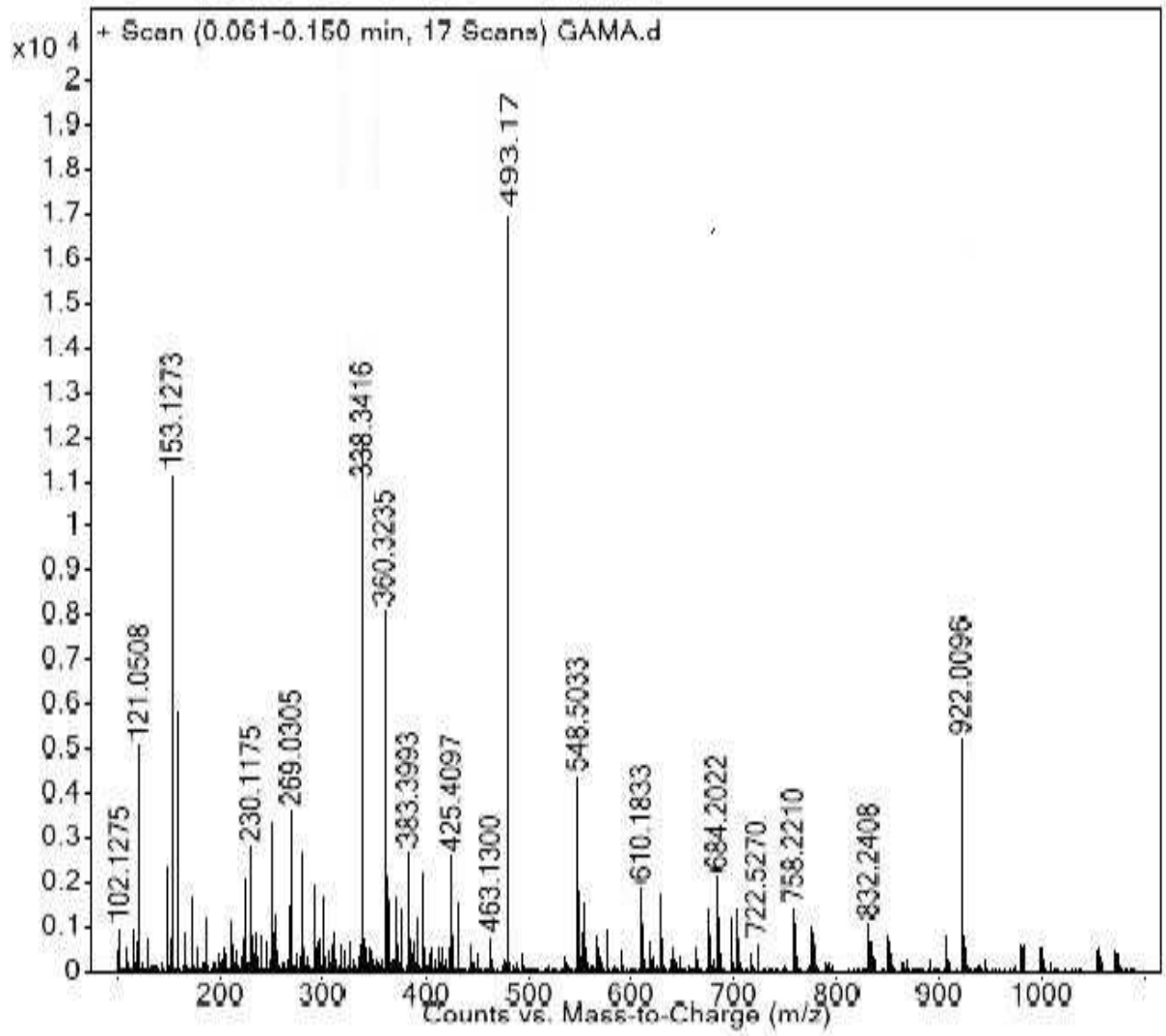


1-N-(4-aminobiphenyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine

Molecular weight- 493

M/z(M+H)=325

AnalysisFileName	AcquiredTime	InstrumentName
GAMA.d	22-Apr-2016	LC-QTOF MS/MS



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8. IN-VITRO SCREENING FOR ANTICANCER ACTIVITY:

8.1. Introduction:

In vitro (Latin: in glass) studies in experimental biology are those that are conducted using components of an organism that have been isolated from their usual biological surroundings in order to permit a more detailed or more convenient analysis than can be done with whole organisms. Commonly, these experiments are called "test tube experiments" or “cell culture”. Now a days, most cell culture is performed in plastic culture plates or petridishes.[1]

The discovery of a new drug requires it to be tested first in-vitro then on the animals before being used on the human being to provide the best possible active agents for the human use with minimum toxicity.

In vitro bioassays are commonly used to screen synthetic organic compounds or natural products for potential cancer therapeutic activity. These assays usually consist of cell culture systems in which neoplastic cell lines have been established from human or other animal tumors. The ability of the tested compounds to inhibit the growth of these cancer cells in culture is taken as an indication of potential value as a cancer therapeutic agent in vivo. In addition, these bioassays are often used in structure- activity studies of a series of structurally related synthetic or semi-synthetic compounds to determine the effects of a particular chemical modification of a reactive moiety of the compound on its biological activity. Because of these uses,it is important to establish the parameters of the assays which will allow the best quantitative comparisons of tested materials.

The MTT Cell Proliferation Assay measures the cell proliferation rate, consequently when metabolic events leads to necrosis or apoptosis, the reduction in the cell viability. The number of assay steps has been minimised as much as possible to expedite sample processing. The MTT reagents yield low background absorbance values in absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus, allowing an accurate quantification of changes in the rate of proliferation.[2]

8.2. MTT assay:

MTT, a yellow tetrazole, is reduced to purple formazan in living cells. A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple

formazan product into a coloured solution. The absorbance of this coloured solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption maximum is dependent on the solvent employed.[3]

The MTT assay is a colorimetric assay for assessing cell viability. NADPH-dependent cellular oxidoreductase enzymes under defined conditions, reveal the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide to its insoluble formazan which has a purple colour. It can be solubilized and quantified by spectrophotometry. MTT dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferative to resting status) of potential medicinal agents and toxic materials. MTT assays are usually done in the dark since the MTT reagent is sensitive to light.[4]

8.3. Precaution:

- ***Handling***

Safe laboratory procedures should be followed when handling all kit reagents. It is recommended that protective laboratory clothing and equipment(gloves,laboratory coat,safety glasses) be worn when handling kit reagents.

- ***Emergency Exposure Procedures***

In case of exposure to reagent solutions, we recommend following these emergency first-aid procedures:

- ***Skin or eye contact***

Wash with water for at least 15 minutes. Remove any contaminated clothing.

- ***Inhalation***

Remove individual to fresh air. If breathing is difficult, give oxygen and call a physician.

- ***Ingestion***

Rinse mouth with copious amounts of water and call a physician.

8.4. MTT assay Experimental Procedure:

The experiments reported hereinwere undertaken in order toinvestigate the cytotoxicity against a panel of Human Leukemia Cell-line (K-562) recommended by National Cancer Institute (NCI) were used for preliminary in-vitro cytotoxicity assay of the synthesized compound.

Work followed during MTT Assay step wise are as follows:

- ***Step-1***

The above mentioned cells were cultured in DMEM supplimented with 10% Foetal Bovine Serum and 1% antibiotic (Gentamycin) at 37 OC in a humidified atmosphere under 5% CO2.

- ***Step-2***

After 75% confluence, cells were harvested with 0.025% trypsin and 0.052 mM EDTA in Phosphate Buffer Solution (PBS).

- ***Step-3***

Harvest suspension cells by centrifugation. Adherent cells should be released from their substrate by trypsinization or scraping, Resuspend , Prepare serial dilutions of cells in culture medium.

- ***Step-4***

The cell media (100 microlitre/wellplates, no of cells/wellplate approximately 5000-10000) were plated in 96 well-plates .

- ***Step-5***

Treated with different concentrations (50-300 uM) of synthesized compounds for 24-48 hours. It include two control wells of medium alone to provide the blanks (Without synthesized compound) for absorbance readings.

- ***Step-6***

Add 10 µL of MTT Reagent to each well, including controls.

- ***Step-7***

Return plate to cell culture incubator for 2 to 4 hours.

- ***Step-8***

Periodically view the cells under an inverted microscope for presence of intracellular punctate purple precipitate.

- ***Step-9***

When the purple precipitate is clearly visible under the microscope add 100 µL of DMSO Reagent to all wells, including controls. Swirl gently, do not shake.

- ***Step-10***

Remove plate cover and measure the absorbance in each well, including the blanks, at 570 nm in a microtiter plate reader.[5]

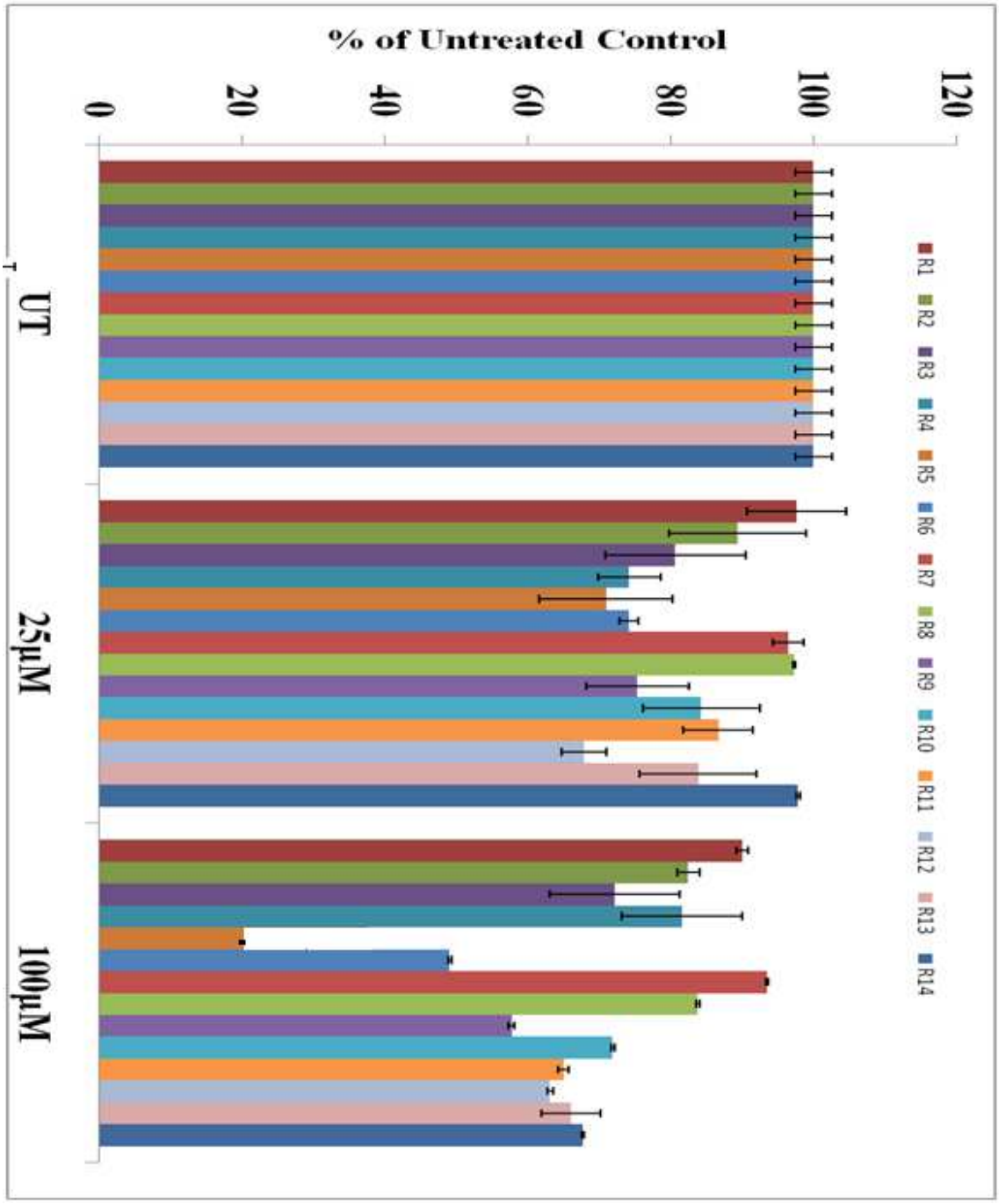
Special Note: *If the readings are low return the plate to the dark for longer incubation. 14 Determine the average values from triplicate readings and subtract the average value for the blank. Plot absorbance against number of cells/mL. The number of cells to use in your assay should lie within the linear portion of the plot and yield an absorbance of 0.75 - 1.25.*

8.5. CELL CYTOTOXIC ACTIVITY OF SYNTHESIZED COMPOUNDS:

SL. NO.	COMPOUNDS	%Cell Viability or IC-50
R1	1-N-Isobutyl-2-N-(4-biphenylacetyl)-L (+)-isoglutamine	503.72µM
R2	1-N-Phenyl-2-N-(4-biphenylacetyl)-L (+)-isoglutamine	303.93µM
R3	1-N-(4-Fluorobenzyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine	184.60µM
R4	1-N-(n-Hexyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine	337.98µM
R5	1-N-3,5,-Bisdi(trifluoromethyl)benzyl-2-N-(4-biphenylacetyl)-L(+)isoglutamine	20.29µM
R6	1-N-(4-Biphenyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine	97.51µM
R7	1-N-(2-Benzoxazolyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine	828.76µM
R8	1-N-(n-Butyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine	305.43µM

R9	1-N-(2-Chlorobenzyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine	114.82µM
R10	1-N-(n-Pentyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine	181.42µM
R11	1-N-(4-Nitrobenzyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine	142.89µM
R12	1-N-(2-Benzimidazolyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine	312.85µM
R13	1-N-(orthoaminophenyl)-2-N-(4-biphenylacetyl)-L (+)-isoglutamine	147.26µM
R14	1-N-Benzyl-2-N-(4-biphenylacetyl)-L(+)-isoglutamine	144.16µM

8.6. Graph of %Cell Viability Assay



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9. Discussion

Cancer results from a series of molecular events that fundamentally alter the normal properties of cells. In cancer cells the normal control systems that prevent cell overgrowth and the invasion of other tissues are disabled. These altered cells divide and grow in the presence of signals that normally inhibit cell growth; therefore, they no longer require special signals to induce cell growth and division.

Cancer has become most deleterious hazards among most of the people worldwide. According to the Global Cancer Report issued by the World Health Organization (WHO), there are 14.1 million new cancer cases per year worldwide and corresponding estimates for total cancer annual deaths 8.2 million from cancer disease.

The biochemical evidence suggests that glutamic acid is present in the products of hydrolysis of malignant tumours which gives the idea of glutamine antagonists as a new class anticancer agent. The fact is that the neoplastic transformation is accompanied with increase in nucleotide and protein synthesis, for which the nitrogen demand of cancerous cells increases tremendously. Glutamine is the most abundant free amino acid in the human body. It carries nearly one third of circulating amino acids along with nitrogen. It is the predecessor for biosynthesis of several biomolecules including purine and pyrimidine bases as well as other amino acids. It is the principle metabolic fuel for rapidly dividing cells including enterocytes, colonocytes, fibroblasts, lymphocytes, macrophages and neutrophils. Glutamine is an essential component in the proliferation of tumour cells. In malignant cells, glutamine level remains very high and tumours incorporate not only the nitrogen from the diet but also from host proteins, raising the concept of tumours as nitrogen traps rather glutamine traps as it is the main vehicle of nitrogen circulation in nontoxic form. A novel approach of cancer chemotherapy is to kill the cancer cells by making them deficient of an important cellular nutrient, glutamine as well as its closely related congener asparagine. If synthesis of glutamine can be stopped selectively in malignant cells then the uncontrolled proliferation can also be controlled. The result will be the death or inhibition of growth of cancer cells due to deficiency of required biomolecules.

Glutamine promotes the hallmarks of malignancy by acting as a continuous source of energy formation, sustaining proliferative signal, enabling replicative mortality, resisting cell growth and increasing invasion and metastasis. If synthesis of glutamine can be stopped selectively in malignant cells then the uncontrolled proliferation can also be controlled. The result will be the

death or inhibition of growth of cancer cells due to deficiency of required biomolecules. It is found that a number of glutamine antagonists (e.g. azaserine, DON, acivacin, chloroketone) have potent anticancer activity.

10. Conclusion

In earlier works, workers in our laboratory developed glutamine antagonists that are structurally glutamine and glutamamide analogs. It is found that many of these have promising cell cytotoxic activity, Matrix metalloproteinase and histone deacetylase blocking better activity specially MMP2 and HDAC8.

In this dissertation, invention of some novel anticancer agents was the target as cancer has become one of the most dreadful disease in the present scenario causing death of huge number of people. The number of cancer patient is increasing day by day and the conventional anticancer drugs are not able to completely cure this disease.

With the perspective of developing better active anticancer agents some novel molecules, in the present work, fourteen isoglutamine analogues i.e, 1-N-substituted-2-N-(substituted biphenylacetyl)-L(+)-isoglutamine analogues were synthesized, characterized and evaluated for their anticancer activity. The synthesis of these compounds was done through rational drug designing approach. The decision of choosing glutamine analogues has already been discussed as many evidences suggests that glutamine plays an important role in the growth and development of tumour cells.

Among the 14 synthesized compounds, 6 compounds (R5, R6, R9, R11, R13, R14) has notable cytotoxic activity against K562 cell-lines (Human Leukemia Cell-line). Out of these 6 better active compounds, 2 compounds less than 100µM(R5 and R6) are more efficacious which has IC50.

On the basis of this conclusion, in future the scope of developing new anti-cancer agents with more potency has increased. Synthesizing,characterising and evaluating the new 1-N-substituted-2-N-(substituted biphenylacetyl)-L(+)-isoglutamine derivatives may lead to new approach towards the anticancer drug development.