Evaluation of cytotoxic and apoptotic potential of anticancer molecule

A Dissertation

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TO WHOM IT MAY CONCERN

This is to certify that **Ms. Priyanka Sharma,** student of M.Sc. Biotechnology from Thapar University, Patiala has successfully completed her project work on **"Evaluation of cytotoxic and apoptotic potential of an anticancer molecule"** in this institute from 07-01-2014 to 10-07-2014.

She is honest, sincere and a hard worker. I wish her all the success in her life.

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CERTIFICATE

I certify that this thesis entitled 'Evaluation of cytotoxic and Apoptotic potential of anticancer molecule' comprises research work carried out by **Privanka Sharma** under my supervision at the Cancer Pharmacology Division, **Indian Institute Of Integrative Medicine**, during the period between **7th January to 7th July 2014** for the degree of **Masters of Science in Biotechnology** from the **Thapar University**. The results presented in this thesis have not been submitted previously to this or any other university for a M.Sc. or any other degree.

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I certify that Priyanka Sharma is a student of the Thapar University and has submitted a thesis entitled "Evaluation of Cytotoxic and Apoptotic Potential of Anticancer molecules" to the Thapar University as a requirement for the degree of Master of Science in Biotechnology.

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DECLARATION

"I hereby declare that the dissertation entitled "Evaluation of cytotoxic and Apoptotic potential of an anticancer molecule" submitted to Thapar Institute of Engineering and Technology in partial fulfillment of the requirements for the award of degree of Masters in Biotechnology, is a record of project work carried out under the guidance of Dr. Shashi Bhushan, department of Cancer Pharmacology, Indian Institute of Integrative Medicine, Jammu and this dissertation or part thereof has not been submitted elsewhere for any other degree.

Priyanka Sharma

Date: 10.07.2014

Jammu

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LIST OF ABBREAVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AIF	Apoptosis Inducing Factor
BSA	Bovine Serum Albumin
CSIR	Council of scientific and Industrial Research
DMSO	Dimethyl Sulfoxide
DISC	Death Inducing Signalling Complex
EGF	Epidermal growth factor
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
HL60	Human Leukemia cancer
HRP	Horse Redish Peroxidase
IIIM	Indian Institute of Integrative Medicine
MQ	Millipore Water
MMP	Mitochondrial Membrane Potential
MEM	Minimal's Eagle media
MTP	Mitochondrial Transisition Pore
MIA-PACA	Pancreatic cancer
MCF7	Breast cancer
PC3	Prostrate cancer
PBS	Phosphate buffered saline
PTP	Permeability Transition Pore
PI	Propidium Iodide Dye
PMS	Poly Methyl Sulfoxide
PV F	Poly Vinylidene Fluoride Membrane

RMI	Rosewell Park Memorial Institute
ROS	Reactive Oxygen Species
RIPA	Radio Immuno Precipitation Assay Buffer
SDS	Sodium Dodecyl Sulphate
TGF	Tumour Growth Factor
TE	Tris EDTA Buffer
TB	Transfer Buffer
TNFR	Tumour Necrosis Factor Receptor
T47D	Breast cancer
W/v	Weight/volume

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Chapter 1

Introduction

Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems. Our bodies are composed of trillions of cells, all working together. In cancer, one of those cells stop paying attention to the normal signals that tell cells to grow, stop growing or even to die. Cancer cells still share many of the same needs and properties of normal cells but they become independent of the controls that make our body function smoothly. The process by which a normal cell changes into one that behaves so abnormally can take a long time and is often triggered by outside influences. Cancer is actually a general term that describes a large group of related diseases. Cancer is the result of unregulated cell division. Cancer cells divide when they are not supposed to, don't stop dividing when they are supposed to and don't die when they should. In the worst cases, the cancer cells leave the area in which they arose and travel to other parts of the body. The cells continue to divide, impacting nearby normal cells, often reducing the function of the affected organ. Cancer is a major public health threatening disease in developed countries (**Balunas, and Kinghorn, 2005, Tan *et al*. 2006**). Cancer is a common condition and is a serious health problem, both in the UK and across the world. It is estimated that 7.6 million people in the world died of cancer in 2007. In the UK, cancer is responsible for 126,000 deaths per year. One in four people die from cancer.

Cancer is the second most common disease after the coronary heart diseases. In another two decades the incidence of cancer is expected to raise world over **(Parkin *et al.*, 1999)**. So it is a great challenge to mitigate this deadly disease through conventional or non-conventional therapeutics. Many approaches have been made in this direction in tackling this disease, but no individual approach has been successful. Drugs developed using past paradigms attack both cancerous and healthy cells, often causing devastating short and long-term side effects. Moreover, individual patient responses to conventional agents vary, even in cases where cancers appear to be identical. Development of a novel chemical entity into a modern drug is often associated with many side effects. This is evident from chemotherapeutic agents, which attack the rapidly dividing cancer cells, but acts in a harmful way upon the structure and function of normal cells, tissues or organ systems of the body.Though many chemotherapeutic agents have been available in the market but almost all

the available drugs have adverse side effects on the normal cells of the body. The major drawback of the most of the chemotherapeutic agents available today is immunosuppressant, cytotoxic to normal cell, causing harm to the body by actively disrupting the vital processes and exert a variety of side effects that are particularly evident in cancer chemotherapy.

So it is a great challenge to design a therapy which is both effective and also has high specificity for the biology of cancer and/or is efficiently targeted to tumor tissue. These agents should ideally be selective for normal cells versus cancer cells, be effective in reducing or preventing toxicity, should have no negative impact on anticancer therapy, and have minimal adverse effects. None of the agents currently under development fulfills these criteria completely **(Hoekman *et al.*, 1999).** In cancer, the therapeutic goal is to trigger tumor-selective cell death. The mechanisms responsible for such death are of obvious importance in determining the efficacy and management of specific treatments. With the discovery that distinct death pathways exist in biology, and that certain of these are evolutionarily selected and highly efficient, there came an explosion of interest in connecting such pathways in the pathophysiology of cancer. Successful drug treatment of cancer or any human disease requires an adequate therapeutic index reflecting the treatment's specific effect on target cells and its lack of clinically significant toxic effect on the host or normal cells. An anticancer drug will achieve a suitable therapeutic index in several ways. First, it might activate a death cascade via a drug target that is uniquely expressed in a cancer cell. Secondly, it might be delivered to the target tissue in a manner that is selective for the cancer cell. A third and perhaps more promising approach is to exploit a pathway that is activated by oncogenes, in order to provoke apoptosis selectively in cancer cells.

Evasion of apoptosis is the hallmark of all cancers, so targeting it provides a bull's eye oriented approach in novel anticancer drug development, which is either from synthetic, semi synthetic or natural sources. Many compounds derived from the nature or other sources may be cytotoxic but selection of only those compounds is desirable which kill the cancer cells by apoptosis rather than necrosis. Inhibition of cancer cell proliferation and induction of apoptosis, are two-desired character of any new molecule for its development as anticancer agents. Apoptosis has been accepted as the predominant mechanism of drug-induced cell death in preclinical experimental models and in clinically sensitive tumors ***(Houghton, 1999)***. Apoptosis is characterized by caspase activation, cell condensation and apoptotic bodies' formation. In early apoptotic cells the membrane remains intact and the cells are rapidly phagocytosed by macrophages or surrounding cells before lysis. This prevents

leakage of cellular contents into the surrounding tissue, making apoptosis a 'clean' cell death process that minimizes the risk of inflammation. Necrosis is an alternative cell death process that is typically caspase-independent and characterized by permeabilization of the plasma membrane and swelling of the cell and its organelles. The release of the contents of necrotic cells may cause damage to the surrounding tissue by inducing an inflammatory reaction.

Nearly all cancers are caused by abnormalities in the genetic material of the transformed cells. These abnormalities may be due to the effects of carcinogens, such as tobacco smoke, radiation, chemicals, or infectious agents. Other cancer-promoting genetic abnormalities may be randomly acquired through errors in DNA replication, or are inherited, and thus present in all cells from birth. The heritability of cancers are usually affected by complex interactions between carcinogens and the host's genome. New aspects of the genetics of cancer pathogenesis, such as DNA methylation, and microRNAs are increasingly recognized as important.

Genetic abnormalities found in cancer typically affect two general classes of genes. Cancer-promoting oncogenes are typically activated in cancer cells, giving those cells new properties, such as hyperactive growth and division, protection against programmed cell death, loss of respect for normal tissue boundaries, and the ability to become established in diverse tissue environments. Tumor suppressor genes are then inactivated in cancer cells, resulting in the loss of normal functions in those cells, such as accurate DNA replication, control over the cell cycle, orientation and adhesion within tissues, and interaction with protective cells of the immune system.

In the continuing search for agents that may treat or ameliorate the affliction of cancer, natural products have provided an endless supply of active compounds that are increasingly being exploited. Indeed, natural products have been the mainstay of cancer chemotherapy over the last three decades **(Da rocha,** ***2001)*** and plant extracts have been used to treat cancers for even longer **(Newman dj** ***et al., 2000)*** . For instance, the chemotherapeutic drugs etoposide and teniposide are derivatives of podophyllotoxin, the chief constituent of the extracts from the roots of mayapple, *Podophyllum peltatum.* These root extracts have been used by generations of American Indians for the treatment of skin cancers. Similarly, the vinca alkaloids, vinblastine and vincristine, are derived from the Madagascar periwinkle plant (Catharanthus *roseus,* formerly known as *Vinca rosea*), which was used as hypoglycemic agent in Asia. Ayurveda, one of the major traditional forms of medical practice in India dating back thousands of years, has used plant-based medicines for cancer prevention **(Garodia*et al., 2007)***. The efficacy of these agents in the treatment of a wide spectrum of

cancers **(Duflos *et al., 2002)*** persuaded the National Cancer Institute to begin a large-scale screening program for antitumor agents from natural sources in the 1960s **(Zubrod CG *et al.,1984)***. The most prominent antitumor agent that emerged from this screening was paclitaxel obtained from the bark of the Pacific yew tree, *Taxus brevifolia.*

Cancer cells cleverly evade self-demise through apoptosis because of the accumulation of several genetic and epigenetic changes within **(G. Klein, 2004)**. Agents that can trigger the process of apoptosis in cancer cells are therefore considered potentially important for the development of anti-cancer chemotherapeutic (**K.H. Lee, 1999)**. Of several prescription drugs in use for cancer treatment, almost 75% are derived from plant species.**(W.J. Craig, 1997).** Most of the current anti-cancer drugs are derived from plant sources, which act through different pathways converging ultimately into the activation of apoptosis in cancer cells leading to cell cytotoxicity **(Lee KH, 1999)**. Anti-neoplastic agents therefore, act through several pathways in the death of cancer cells. Recent studies have amply documented that two major pathways are involved in the regulation of apoptosis **(Earnshaw WC *et al.,* 1999)**. One pathway is mediated via cell surface death receptors, such as Fas/CD95 and TNFR1, which upon activation recruit cytoplasmic tail of the receptors and downstream associated signaling complex leading to the activation of caspase-8. The second pathway is mitochondrial-dependent, which is regulated by signaling cascade-involving members of the Bcl-2 family. A loss of mitochondrial membrane potential brings about the translocation of proapoptotic Bax to mitochondria and cytochrome c from mitochondria to cytosol resulting in caspase-9 activation (**Jiang X, Wang X, 2000)**. Members of the Bcl-2 family therefore play a crucial role in the regulation of apoptosis. For instance, overexpression of anti-apoptotic Bcl-2 prevents the release of cytochrome c, while over expression of pro-apoptotic Bcl-2 member Bax facilitates the formation of mitochondrial pores and release of cytochrome c **(Finucane DM *et al.,* 1999)** after depolarization of mitochondrial membranes **(Borner C, 2006)**. Many anti-cancer drugs would act as president, which target mitochondria Green **(DR, Reed JC, 1998)** and may initially involve generation of free radicals such as reactive oxygen/ nitrogen species **(Shen HM, Liu ZG, 2006)** eventually leading to the activation of apoptosis. Currently, natural plant based products are increasingly investigated for their cytotoxicity in cancer cells targeting apoptosis activation for the development of anti-cancer leads.

1.2 TYPES OF CANCER

Carcinoma: A tumor derived from epithelial cells, those cells that line the surface our skin and organs. Our digestive tract and airways are also lined with epithelial cells. This is the most common cancer type and represents about 80-90% of all cancer cases reported.

Sarcoma: A tumor derived from muscle, bone, cartilage, fat or connective tissues.

Leukemia: A cancer derived from white blood cells or their precursors. The cells that form both white and red blood cells are located in the bone marrow.

Lymphoma: A cancer of bone marrow derived cells that affect the lymphatic system.

Myelomas: A cancer involving the white blood cells responsible for the production of antibodies (B lymphocytes or B-cells).

Central nervous system cancers: Cancer that begins in the tissues of the brain and spinal cord.

1.2.1 STAGES OF TUMOR PROGRESSION:

Cancerous cells develop from healthy cells in a complex process called malignant transformation.

Initiation: The first step in cancer development is initiation, in which a change in the cell's genetic material primes the cell to become cancerous. The change in the cell's genetic material may occur spontaneously or be brought on by an agent that causes cancer (a carcinogen). Carcinogens include many chemicals, tobacco, viruses, radiation, and sunlight. However, not all cells are equally susceptible to carcinogens. A genetic flaw in a cell may make it more susceptible.

Promotion: The second step in the development of cancer is promotion. Agents that cause promotion, or promoters, may be substances in the environment or even some drugs (such as barbiturates). Unlike carcinogens, promoters do not cause cancer by themselves. Instead, promoters allow a cell that has undergone initiation to become cancerous. Promotion has no effect on cells that have not undergone initiation. Thus, several factors, often the combination of a susceptible cell and a carcinogen, are needed to cause cancer.

Some carcinogens are sufficiently powerful to be able to cause cancer without the need for promotion. For example, ionizing radiation (which is used in x-rays and is produced in nuclear power plants and atomic bomb explosions) can cause various cancers, particularly sarcomas, leukemia, thyroid cancer, and breast cancer.

Metastasis: Cancer can grow directly into surrounding tissue or spread to tissues or organs, nearby or distant. Cancer can spread through the lymphatic system. This type of spread is typical of carcinomas. For example, breast cancer usually spreads first to the nearby lymph

nodes, and only later does it spread to distant sites. Cancer can also spread via the bloodstream. This type of spread is typical of sarcomas.

1.2.2 CANCER DEVELOPMENT:

A compound that reacts with DNA and somehow changes the genetic makeup of the cell is called a mutagen. The mutagens that predispose cells to develop tumors are called initiators and the non-reactive compounds that stimulate tumor development are called promoters. Approximately 70% of known mutagens are also carcinogens--cancer-causing compounds. A compound that acts as both an initiator and a promoter is referred to as a 'complete carcinogen' because tumor development can occur without the application of another compound.

The Cancer development constitutes the following:-

Initiators and Promoters: Initiation is the first step in the two-stage model of cancer development. Initiators cause irreversible changes (mutations) to DNA that increase cancer risk. Promotion is the second step in the two-stage model of cancer development. Once a cell has been mutated by an initiator, it is susceptible to the effects of promoters. Promoters increase the proliferation of cells and there are two main types:-

Carcinogens: Substances which can cause cancer are known as carcinogens. The process of cancer development is called carcinogenesis. Certain carcinogenic chemicals are associated with an increased risk of specific cancers due to chronic exposure. One of the most potent carcinogens in humans is benzo[a]pyrene, a compound found in cigarette smoke.

Chronic Inflammation: Chronic inflammation is an important factor in tumor development. Inflammation can lead to altered behavior of cells, stimulation of blood vessel growth (angiogenesis) and tissue remodeling. Markers of inflammation correlate with a worse prognosis for cancer patients.

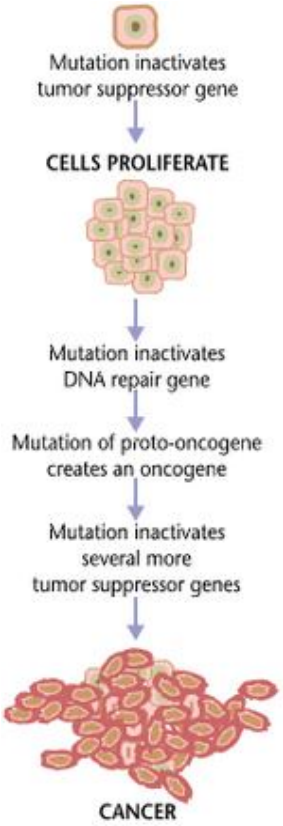


Fig 1 mechanism of cancer cell formation

1.2.3 CANCER TREATMENT:

The treatment given for cancer is highly variable and dependent on a number of factors including the type, location and amount of disease and the health status of the patient. The treatments are designed to either directly kill/remove the cancer cells or to lead to their eventual death by depriving them of signals needed for cell division. Other treatments work by stimulating the body's own defenses.

Cancer treatment is one of the most complex aspects of medical care. It involves surgery, radiation therapy, chemotherapy, targeted therapies, biological therapy and hormonal therapy. In most of the cases one or more treatment modalities may be used to provide the most effective treatment. Increasingly, it is common to use several treatment modalities together (concurrently) or in sequence with the goal of preventing recurrence. This is referred to as multi-modality treatment of the cancer. Once diagnosed, cancer is usually treated with a combination of surgery**,** chemotherapy and radiotherapy. Advances in science and technology have led to the development of several different types of therapies each of these therapy

targets cancers through different mechanisms. Some of the main cancer therapies are given below:

- **Surgery**: In theory, cancers can be cured if entirely removed by surgery, but this is not always possible. When the cancer has metastasized to other sites in the body prior to surgery, complete surgical excision is usually impossible. Examples of surgical procedures for cancer include mastectomy for breast cancer and prostatectomy for prostate cancer. The goal of the surgery can be either the removal of only the tumor, or the entire organ **(Song *et al.*, 2008).**

- **Radiation therapy:** Radiation therapy (also called radiotherapy, X-ray therapy, or irradiation) is the use of ionizing radiation to kill cancer cells and shrink tumors **(Siegel *et al.*, 2008).** The effects of radiation therapy are localized and confined to the region being treated. Radiation therapy injures or destroys cells in the area being treated (the "target tissue") by damaging their genetic material, making it impossible for these cells to continue to grow and divide. Radiation therapy may be used to treat almost every type of solid tumor, including cancers of the brain, breast, cervix, larynx, lung, pancreas, prostate, skin, stomach, uterus, or soft tissue sarcomas.

- **Chemotherapy:** Chemotherapy is the treatment of cancer with drugs that can destroy cancer cells. In current usage, the term "chemotherapy" usually refers to cytotoxic drugs which affect rapidly dividing cells in general, in contrast with targeted therapy **(Kozubík *et al.*, 2008).** Chemotherapy drugs interfere with cell division in various possible ways, e.g. with the duplication of DNA or the separation of newly formed chromosomes. Most forms of chemotherapy target all rapidly dividing cells and are not specific for cancer cells. Hence, chemotherapy has the potential to harm healthy tissue, especially those tissues that have a high replacement rate (e.g. intestinal lining).

- **Targeted therapies:** Targeted therapy, which first became available in the late 1990s, has had a significant impact in the treatment of some types of cancer, and is currently a very active research area. This constitutes the use of agents specific for the deregulated proteins of cancer cells. Small molecule targeted therapy drugs are generally inhibitors of enzymatic domains on mutated, over expressed or otherwise critical proteins within the cancer cell. Prominent examples are the tyrosine kinase inhibitors imatinib and gefitinib.

- **Photodynamic therapy (PDT)** is a ternary treatment for cancer involving a photosensitizer, tissue oxygen, and light (often using lasers). PDT can be used as treatment for basal cell carcinoma (BCC) or lung cancer; PDT can also be useful in removing traces of malignant tissue after surgical removal of large tumors **(Dolmans, 2003).**

- **Immunotherapy:** Cancer immunotherapy refers to a diverse set of therapeutic strategies designed to induce the patient's own immune system to fight the tumor. Contemporary methods for generating an immune response against tumours include intravesical BCG immunotherapy for superficial bladder cancer, and use of interferons and other cytokines to induce an immune response in renal cell carcinoma and melanoma patients **(Ortiz-Sánchez *et al.*, 2008).**

- **Hormonal therapy:** The growth of some cancers can be inhibited by providing or blocking certain hormones. Common examples of hormone-sensitive tumors include certain types of breast and prostate cancers **(Barlow *et al.*, 2008).** Removing or blocking estrogen or testosterone is often an important additional treatment. In certain cancers, administration of hormone agonists, such as progestogens may be therapeutically beneficial.

- **Complementary and alternative medicine:** Complementary and alternative medicine (CAM) treatments are the diverse group of medical and health care systems, practices, and products that are not part of conventional medicine **(Smith *et al.*, 2008).** Many "complementary" and "alternative" medicines for cancer have not been studied using the scientific method, such as in well-designed clinical trials, or they have only been studied in preclinical (animal or in-vitro) laboratory studies **(Wesa *et al.*, 2007).**

Vaccines: The purpose of cancer vaccines is to stimulate the body's defenses against cancer. Vaccines usually contain proteins found on or produced by cancer cells. By administering these proteins, the treatment aims to increase the response of the body against the cancer cells.

1.3 MEDICINAL PLANTS

The plant kingdom has always been the favorite source of medication in all-healing traditions all over the world. The use of plants as medicine is as old as human civilization, but with the faster pace of life and the need for rapid cure laid the proliferation of synthetic drugs, however, with those drugs came the problems of side effects, ill effects and complications. Today, the wheel has turned a full circle with the medical and scientific fraternity being involved in the study and development of safe and effective natural drugs which are mainly of plant origin. **(Parenjpel, 2001)**. In recent times, focus on plant research has greatly increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems. India in this regard has a unique position in the world, where a number of traditional systems of medicine, viz, Ayurveda, Siddha, Unani are practiced and utilized in the total health care system of the country and all these systems are predominantly dependent upon medicinal plants.

Recent observations indicate that perhaps 80% of the world's population rely solely upon medicinal plants for the treatment of diseases. The goals of selection plants as sources of therapeutic agents are to isolate bioactive compounds for direct use as drugs, e.g., digoxin, digitoxin, morphine, reserpine, Taxol, vinblastine, vincristine; to produce bioactive compounds of novel structures as lead compounds for semi synthesis and to produce patentable entities of higher activity and lower toxicity. In the opinion of the experts it is considered that the management of cancer is still not up to the mark and thus, there is definitely a need to search drugs for the management of cancer. In this context, the majority of most useful and curative drugs, e.g., Taxol from Taxus brevifolia and Taxus baccata for cancer continue to be derived from plant sources.

In view of the above, it is clear that we are in urgent need of drugs for the treatment of cancer that are less toxic and more potent, and medicinal plants have real significance and there is a need to screen Indian medicinal plants for their anticancer activity in vitro and in vivo, and carry out systematic bioassay guided fractionation on them.

1.3.1 Targets of Anti-cancer therapy

In order to eradicate this dreadful disease which is a main cause of human suffering, multi-pronged strategies are designed which involve targeted based chemotherapy utilizing modern bio-informatics tools for drug designing, immunotherapy using designer cancer vaccines based on tumor associated cell surface antigens, anti-angiogenesis therapy and development of tumor specific vehicles for drugs. Modern pharmacological techniques shall be employed for exploring the mechanistic details of drug action. The research spans across several

disciplines viz., chemistry, bio-informatics, pharmacology and immunology and proteomics and clinical pharmacology to take the project to its meaningful deliverables.

Molecularly targeted interventions result from the integration of multiple research disciplines can be classified into five general molecularly targeted strategies that will guide future research to discover and develop new anticancer agent (s) that specifically target the cancer cells without affecting normal cells (Fig 2). Targeting these events should have potent and specific therapeutic consequences.

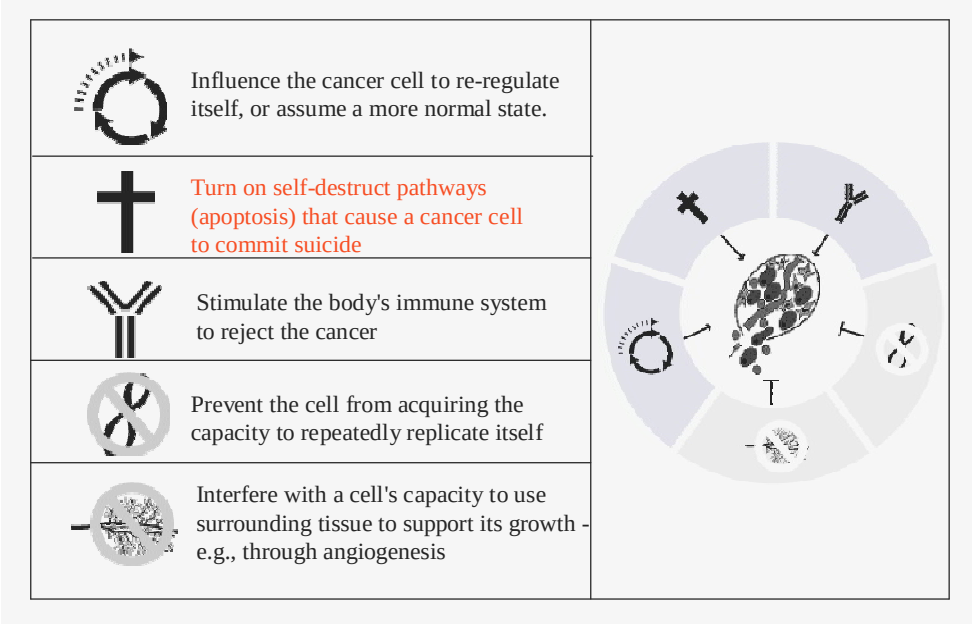


Fig 2: Molecular targets for anti-cancer therapy

One of these events in cell deregulation is obligate compensatory suppression of apoptosis (programmed cell death, PCD), which provides support for neoplastic progression. In this proposal we have evolved a holistic molecular approach based on the fact that several genes are mutated in cancer cells which evade cancer cells from self demise (apoptosis). Whatever the mechanisms involved, if the test compound induces apoptosis that test compound is the potential candidate for anti-cancer lead optimization.

1.3.2 DEVELOPMENT OF ANTICANCER DRUGS FROM PLANTS & MICROBES

It is estimated that there are roughly 500,000 higher flowering plant species occupying terrestrial habitats. A large number of species have only been very superficially examined for their pharmacological and medical application. Less than 1% of these species have been thoroughly investigated for their potential use as novel therapeutic agents.

Traditionally, cancer drugs were discovered through large-scale screening of synthetic chemicals against animal tumor systems, primarily murine leukemias. The agents discovered

in the first two decades of cancer chemotherapy **(1950-1970)** largely interacted with the DNA or its precursors, inhibiting the synthesis of new genetic material or causing irreparable damage to DNA itself. In the area of cancer treatment, many claims have been made for the beneficial effects of plants **(Hartwell. 1982).**

Drug discovery from medicinal plants has played an important role in the treatment of cancer. Of all available anticancer drugs between 1940 and 2002, 40% were natural products per se or natural product-derived with another 8% considered natural product mimics **(Newman et. al., 2003).**

1.4 Apoptosis And Cancer

Apoptosis is a normal component of the development and health of multicellular organisms. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated way. Apoptosis is distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Apoptosis, in fact, is a process in which cells play an active role in their own death that's why it is often referred to as cell suicide. During the normal development of multicellular organisms, apoptosis occur and continues throughout adult life. The combination of apoptosis and cell proliferation is responsible for shaping tissues and organs in developing embryos. This process of apoptosis is also an important part of the regulation of the immune system. T lymphocytes are cells of the immune system that are responsible for destroying infected or damaged cells in the body. They mature in the thymus, but before they can enter the bloodstream they are tested to ensure that they are effective against foreign antigens and are also not reactive against normal, healthy cells. Any ineffective or self-reactive T-cells are removed through the induction of apoptosis **(Lowe et al., 2000)**. A number of mechanisms have been introduced through which apoptosis can be induced in cells. The sensitivity of cells to any of these stimuli can vary depending on a number of factors such as the expression of pro- and anti-apoptotic proteins (eg. the Bcl-2 proteins or the Inhibitor of Apoptosis Proteins), the severity of the stimulus and the stage of the cell cycle. Some of the major stimuli that can induce apoptosis include virus infection, cell stress and DNA damage. In some cases, the apoptotic stimuli comprise extrinsic signals such as the binding of death inducing ligands to ell surface receptors called death receptors **(Sprick *et al*., 2004).**

1.4.1 Molecular mechanisms of apoptosis signaling pathways:

Various death signals activate common signaling pathways

Apoptosis is a tightly regulated and at the same time highly efficient cell death program which requires the interplay of a multitude of factors. The components of the apoptotic signaling network are genetically encoded and are considered to be usually in place in a nucleated cell ready to be activated by a death-inducing stimulus **(Ishizaki, 1995; Weil, 1996).**

Apoptosis can be triggered by various stimuli from outside or inside the cell, e.g. by ligation of cell surface receptors, by DNA damage as a cause of defects in DNA repair mechanisms, treatment with cytotoxic drugs or irradiation, by a lack of survival signals, contradictory cell cycle signaling or by developmental death signals. Death signals of such diverse origin nevertheless appear to eventually activate a common cell death machinery leading to the characteristic features of apoptotic cell death.

Much of the understanding of cell death has come from genetic studies in the nematode C. elegans by which several genes have been identified that function in the apoptotic killing and elimination of 131 of the initially 1090 somatic cells that are generated during hermaphrodite development **(Hengartner, 1999).** The proximal cause of apoptosis in C. elegans is the activation of the cysteine protease ced-3, which is mediated by its oligomerization at the activator protein ced-4. Activity of the ced-3/ced-4 complex is regulated by the apoptosis inhibitor ced-9 and the apoptosis inducer egl-1 (**Fig. 3**). Subsequent studies in mammals and in the fly, D. melanogaster, have identified counterparts for these C. elegans genes, demonstrating that the core components of the cell death machinery are conserved through evolution [Richardson, 2002]. Accordingly, ced-3 is the single C. elegans member of a family of cysteine proteases, the caspases, whereas ced-4 corresponds to the mammalian apoptotic protease activating factor 1, Apaf-1, which is the core of a caspase-activating signaling complex, the apoptosome. Egl-1 and ced-9 are members of the Bcl-2 family of pro- or anti-apoptotic proteins, respectively, which play an important role in the mediation and regulation of apoptotic signaling pathways. All of those central components will be discussed within the following paragraphs.

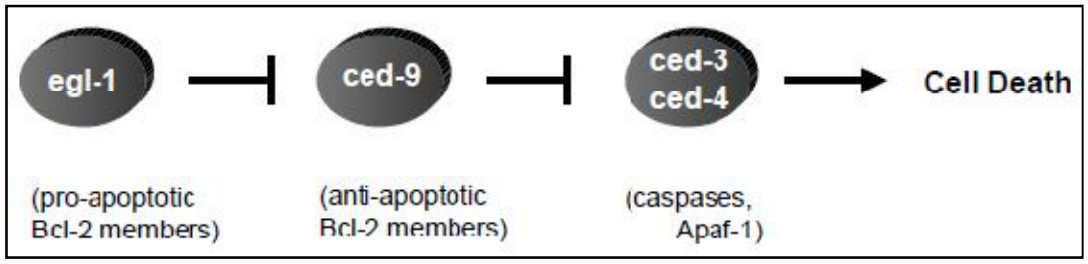


Fig. 3. C. elegans as a model system contains basic components of the cell death machinery. Apoptosis regulation in C. elegans relies on a simple basic network of factors for which corresponding analogous components also can be found in higher organisms as given within brackets. Thus egl-1 is the worm representative for mammalian pro-apoptotic BH3-only proteins, ced-9 belongs to the anti-apoptotic Bcl-2family, ced-3 is the only worm caspase, and ced-4 is homologous to mammalian Apaf-1 (according to **(Cecconi, 1999).**

1.4.2 Caspases are central initiators and executioners of apoptosis

The caspases, cysteine proteases homologous to C. elegans ced-3, are of central importance in the apoptotic signalling networks which are activated in most cases of apoptotic cell death [Bratton, 2000]. Actually, strictly defined, cell death only can be classified to follow a classical apoptotic mode if execution of cell death is dependent on caspase activity **(Leist, 2001).**

The term caspases is derived from cysteine-dependent aspartate-specific proteases: their catalytical activity depends on a critical cysteine-residue within a highly conserved active-site pentapeptide QACRG, and the caspases specifically cleave their substrates after Asp residues. So far, 7 different caspases have been identified in Drosophila, and 14 different members of the caspase-family have been described in mammals, with caspase-11 and caspase–12 only identified in the mouse **(Denault, 2002; Richardson, 2002).** According to a unified nomenclature, the caspases are referred to in the order of their publication: caspase-1 is ICE (Interleukin-1ß-Converting Enzyme), the first mammalian caspase described to be a homologue of Ced-3 [Creagh, 2001; Miura, 1993]. Caspase-1 as well as caspases-4, -5, -11, and –12 appear to be mainly involved in the proteolytic maturation of pro-inflammatory cytokines such as pro-IL-1ß and pro-IL-18 and their contribution to the execution of apoptosis remains questionable [Denault, 2002]. Indeed, mice deficient for caspase-1 or caspase-11 develop normally and cells from those knockout mice remain sensitive to various death stimuli **(Li, 1995; Wang, 1998).** In contrast, gene knockout experiments targeting caspase-3 and caspase-9 resulted in perinatal mortality as a result of severe defects in brain development **(Kuida, 1998; Kuida, 1996),** whereas caspase-8 deficient embryos died after

day 12 **(Varfolomeev, 1998).** This and the observation that cell lines derived from those knockout experiments are resistant to distinct apoptosis stimuli underlines the importance of caspases as proapoptotic mediators. Indeed, caspase-3, caspase-9, caspase-8, and additionally caspases-2, -6, -7, and –10 have been recognized to play an important role in the apoptotic signalling machinery **(Earnshaw, 1999).**

In the cell, caspases are synthesized as inactive zymogens, the so called procaspases, which at their N-terminus carry a prodomain followed by a large and a small subunit which sometimes are separated by a linker peptide. Upon maturation, the procaspases are proteolytically processed between the large and small subunit, resulting in a small and a large subunit. The prodomain is also frequently but not necessarily removed during the activation process. A heterotetramer consisting of each two small and two large subunits then forms an active caspase. The proapoptotic caspases can be divided into the group of initiator caspases including procaspases-2, -8, -9 and –10, and into the group of executioner caspases including procaspases-3, -6, and –7. Whereas the executioner caspases possess only short prodomains, the initiator caspases possess long prodomains, containing death effector domains (DED) in the case of procaspases-8 and –10 or caspase recruitment domains (CARD) as in the case of procaspase-9 and procaspase-2. Via their prodomains, the initiator caspases are recruited to and activated at death inducing signalling complexes either in response to the ligation of cell surface death receptors (extrinsic apoptosis pathways) or in response to signals originating from inside the cell (intrinsic apoptosis pathways).

In extrinsic apoptosis pathways (**Fig. 4**), e.g. procaspase-8 is recruited by its DEDs to the death inducing signalling complex (DISC), a membrane receptor complex formed following to the ligation of a member of the tumor necrosis factor receptor (TNFR) family [Sartorius, 2001]. When bound to the DISC, several procaspase-8 molecules are in close proximity to each other and therefore are assumed to activate each other by autoproteolysis **(Denault, 2002).**

Intrinsic apoptosis pathways (**Fig. 5**) involve procaspase-9 which is activated downstream of mitochondrial proapoptotic events at the so called apoptosome, a cytosolic death signalling protein complex that is formed upon release of cytochrome c from the mitochondria **(Salvesen, 2002b).** In this case it is the dimerization of procaspase-9 molecules at the Apaf-1 scaffold that is responsible for caspase-9 activation **(Denault, 2002).** Once the initiator caspases have been activated, they can proteolytically activate the effector procaspases-3, -6, and -7 which subsequently cleave a specific set of protein substrates, including procaspases

themselves, resulting in the mediation and amplification of the death signal and eventually in the execution of cell death with all the morphological and biochemical features usually observed **(Earnshaw, 1999).**

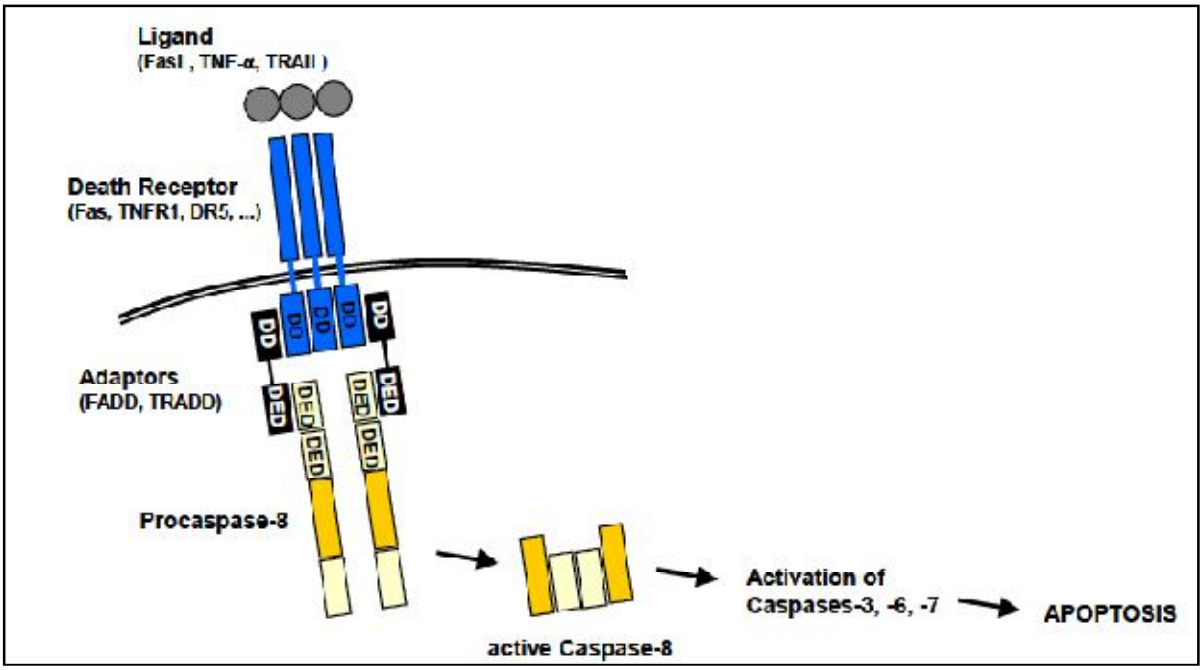


Fig. 4. Receptor-mediated caspase activation at the DISC. Upon ligation by its cognate ligand, thetrimeric death receptor recruits adaptor molecules via its cytoplasmic death domains (DD). Besides possessing DDs, the adaptors additionally contain death effector domains (DED) which recruitprocaspase-8 to the receptor complex which now is called the death-inducing signaling complex(DISC). Procaspase-8 is activated by auto proteolytic cleavage and forms the active caspase-8which is a heterotetramer of two small and two large subunits. The initiatior caspase-8 cleaves and thereby activates effector caspases for the execution of apoptosis.

1.4.3 Extrinsic apoptosis pathways of type I and type II

Extrinsic apoptosis signaling is mediated by the activation of so called “death receptors” which are cell surface receptors that transmit apoptotic signals after ligation with specific ligands. Death receptors belong to the tumor necrosis factor receptor (TNFR) gene super family, including TNFR-1,

Fas/CD95, and the TRAIL receptors DR-4 and DR-5 **(Ashkenazi, 2002)**. All members of the TNFR family consist of cysteine rich extracellular subdomains which allow them to recognize their ligands with specificity, resulting in the trimerization and activation of the

respective death receptor **(Naismith, 1998).** Subsequent signalling is mediated by the cytoplasmic part of the death receptor which contains a conserved sequence termed the death domain (DD). Adapter molecules like FADD or TRADD themselves possess their own DDs by which they are recruited to the DDs of the activated death receptor, thereby forming the so-called death inducing signaling complex (DISC). In addition to its DD, the adaptor FADD also contains a death effector domain (DED) which through homotypic DED-DED interaction sequesters procaspase-8 to the DISC (**Fig. 4**). As described above, the local concentration of several procaspase-8 molecules at the DISC leads to their autocatalytic activation and release of active caspase-8. Active caspase-8 then processes downstream effector caspases which subsequently cleave specific substrates resulting in cell death. Cells harboring the capacity to induce such direct and mainly caspase-dependent apoptosis pathways were classified to belong to the so called type I cells **(Scaffidi, 1998).**

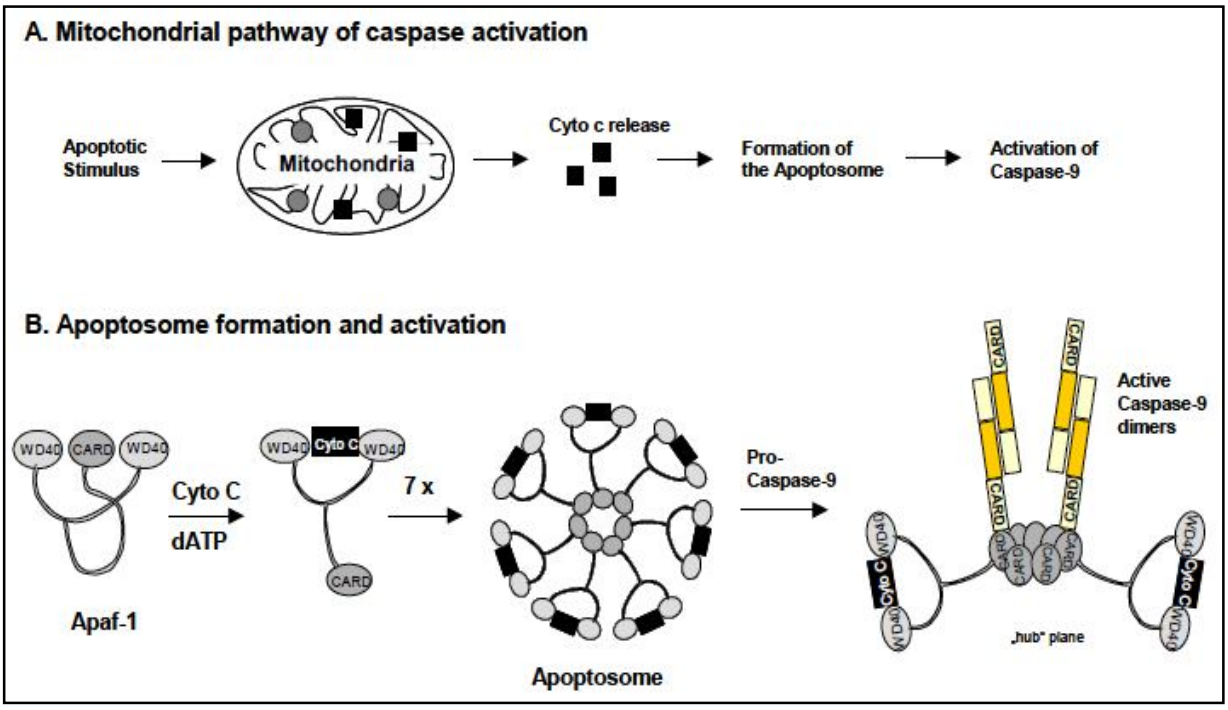


Fig.5 Mitochondria-mediated caspase activation at the apoptosome. A. Apoptotic stimuli trigger therelease of apoptogenic factors from the mitochondrial inter-membrane space to the cytosol, suchas cytochrome c which induces the formation of the apoptosome and the activation of procaspase-9. B. By the action of cytochrome c (Cyto C) and dATP the Apaf-1 protein adopts a conformationthat allows the formation of a heptameric, wheel-like structure, the apoptosome. Procaspase-9molecules can bind to the

inner “hub” region of the apoptosome and are activated by dimer formation. Active caspase-9 dimers further mediate activation of effector caspases **(Acehan,2002).**

In type II cells, the signal coming from the activated receptor does not generate a caspase signalling cascade strong enough for execution of cell death on its own. In this case, the signal needs to be amplified via mitochondria-dependent apoptotic pathways. The link between the caspase signalling cascade and the mitochondria is provided by the Bcl-2 family member Bid. Bid is cleaved by caspase-8 and in its truncated form (tBID) translocates to the mitochondria where it acts in concert with the proapoptotic Bcl-2 family members Bax and Bak to induce the release of cytochrome c and other mitochondrial proapoptotic factors into the cytosol **[Luo, 1998]).** Cytosolic cytochrome c is binding to monomeric Apaf-1 which then, in a dATP-dependent conformational change, oligomerizes to assemble the apoptosome, a complex of wheel-like structure with 7-fold symmetry, that triggers the activation of the initiator procaspase-9 **[Acehan, 2002]**). Activated caspase-9 subsequently initiates a caspase cascade involving downstream effector caspases such as caspase-3, caspase-7, and caspase-6, ultimately resulting in cell death **(Slee, 1999).**

1.4.4 Mitochondria as central regulators of intrinsic apoptosis pathways

Besides amplifying and mediating extrinsic apoptotic pathways, mitochondria also play a central role in the integration and propagation of death signals originating from inside the cell such as DNA damage, oxidative stress, starvation, as well as those induced by chemotherapeutic drugs **(Kaufmann, 2000; Wang, 2001)**. Most apoptosis-inducing conditions involve the disruption of the mitochondrial inner transmembrane potential ($\Delta\psi$) as well as the so called permeability transition (PT), a sudden increase of the inner mitochondrial membrane permeability to solutes with a molecular mass below approximately 1.5 kDa. Concomitantly, osmotic mitochondrial swelling has been observed by influx of water into the matrix with eventual rupture of the outer mitochondrial membrane, resulting in the release of pro apoptotic proteins from the mitochondrial intermembrane space into the cytoplasm **(Bernardi, 1999; Loeffler, 2000)**. Released proteins include cytochrome c, which activates the apoptosome and therefore the caspase cascade, but also other factors such as the apoptosis-inducing factor AIF **(Susin, 199)**, the endonuclease endoG **(Li, 2001),** Smac/Diablo **(Verhagen, 2000),** and Htr/Omi **(Verhagen, 2002).**

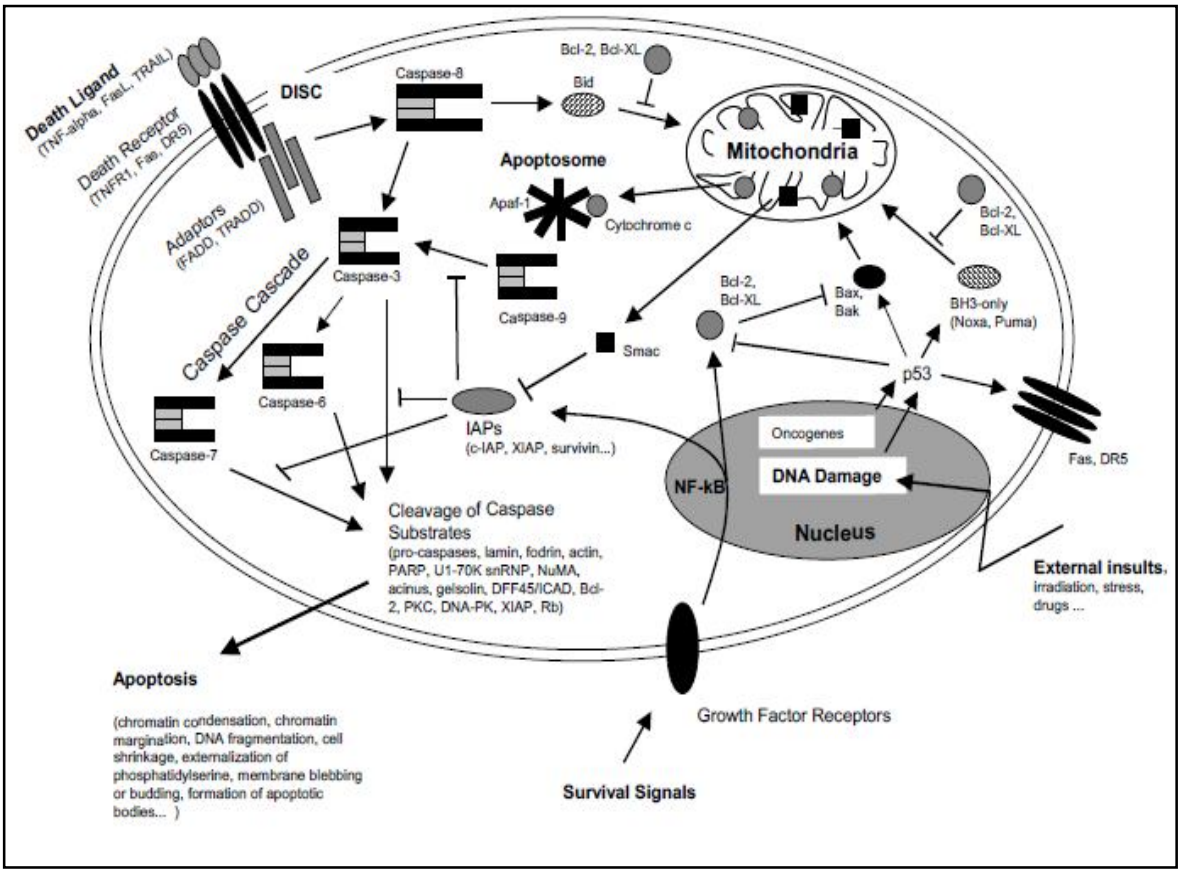


Fig. 6. Schematic representation of some major apoptotic signaling pathways. Apoptosis can be induced in response to various signals from inside and outside the cell, e.g. by ligation of so called death receptors or by cellular stress triggered by oncogenes, irradiation or drugs. Signals emanating from death receptors initially activate the Death Inducing Signaling Complex (DISC) which mediates activation of the initiator caspase-8. Activated caspase-8 initiates a caspase cascade by processing the effector caspases-3, -6, and – 7 which in turn cleave a number of protein substrates. Cleavage ofcaspase substrates eventually leads to the characteristic morphological and biochemical features of apoptosis. In some cell systems, this direct caspase cascade is sufficient to elicit apoptosis on its own(type 1 signaling), whereas in other cases the signal coming from the DISC must be amplified by theproteolytic activation of the BH3-only protein Bid by caspase-8 with subsequent induction of apoptotic events at the mitochondria (type 2 signaling). Mitochondrial apoptotic signaling includes the release of cytochrome c from the mitochondrial inter membrane space to the cytosol where it contributes to the formation of the apoptosome which consists of cytochrome c, Apaf-1 and dATP. The apoptosomeactivates caspase-9 which is another initiator caspase and thus is able to mediate the caspase cascade by activating caspase-3. Another mitochondrial proapoptotic factor is Smac which acts by inhibiting the IAPs from blocking caspase activity. IAPs are a family of proteins with anti-apoptotic activity by directly inhibiting caspases. IAP expression can be upregulated in response to survivalsignals such as those coming from growth factor receptors, e.g. by activation of the transcription factor NF-kB, therefore providing a means to suppress apoptosis signaling. Of central importance are theantiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-XL which counteract the action of BH3-onlyproteins such as Bid but also of pro-apoptotic Bax and Bak and thus can inhibit

mitochondrial proapoptotic events. Apoptotic signals coming from the inside of the cell frequently have their origin within the nucleus, being a consequence of DNA damage induced by irradiation, drugs or other sort of stress. DNA damage in most cases eventually results in the activation of the p53 transcription factor which promotes expression of pro-apoptotic Bcl-2 members and suppresses anti-apoptotic Bcl-2 and Bcl-XL. Other organelles besides mitochondria and the nucleus, such as the ER and lysosomes also have been implicated in apoptotic signaling pathways, and it should be kept in mind that presumably hundreds of proteins are part of an extremely fine-tuned regulatory network consisting of pro- and anti-apoptotic factors.

Interestingly, PT is always followed by $\Delta\psi$, but $\Delta\psi$ is not always caused by PT, and cytochrome c release has been observed even in absence of $\Delta\psi$ **(Bernardi, 1999; Kroemer, 2000)**. In addition to the release of mitochondrial factors, the dissipation of $\Delta\psi$ and PT also cause a loss of the biochemical homeostasis of the cell: ATP synthesis is stopped, redox molecules such as NADH, NADPH, and glutathione are oxidized, and reactive oxygen species (ROS) are increasingly generated **(Kroemer, 2000; Kroemer, 1997).** Increased levels of ROS directly cause the oxidation of lipids, proteins, and nucleic acids, thereby enhancing the disruption of $\Delta\psi$ as part of a positive feedback **(Marchetti, 1997)**. Several possible mechanisms for PT have been proposed, but there appears to exist consent that a so-called permeability transition pore (PTP) is formed consisting of the adenin nucleotide translocator (ANT) and the voltage-dependent anion channel (VDAC) as its core components. ANT is the most abundant protein of the inner mitochondrial membrane and as a transmembrane channel is responsible for the export of ATP in exchange with ADP (antiport). Overexpression of ANT-1 in human cancer cell lines dominantly induces apoptosis with all its characteristic features whereas its closely conserved homologue ANT-2 does not, indicating a specific mechanistic role of ANT-1 in mitochondrial apoptosis events [Bauer, 1999]. VDAC, also called porin, is the most abundant protein of the outer mitochondrial membrane and forms a non-selective pore through the outer membrane. Indicated by direct protein-protein interactions, VDAC-ANT complexes presumably connect inner and outer mitochondrial membrane to so-called ‘contact sites’, corresponding to a close association of the two membranes and thereby possibly constituting the PT pore **(Beutner, 1998).** Since PT, loss of $\Delta\psi$, and release of mitochondrial proteins are of central importance in mediating and enhancing apoptotic pathways, those mitochondrial events must be kept under strict control of regulatory mechanisms which are in many ways dependent on members of the Bcl-2 family.

1.4.5 Regulatory mechanisms in apoptosis signaling:

Commonly, the activation of apoptosis is regarded to occur when a cell encounters a specific death-inducing signal such as the ligation of a death receptor by its cognate ligand or if cells are treated with a cytotoxic drug. This suggests that the apoptosis signaling pathways in viable cells are kept in an inactive state and are only turned on in response to a death stimulus. But it should be taken into account that the components of the apoptotic signaling network are genetically encoded and ready for action in most cell types. Therefore, an interesting and possibly more realistic alternative view would be as follows: all cells of a multicellular animal might be intrinsically programmed to self-destruct and indeed would die instantaneously unless cell death is continuously repressed by survival signals such as provided by other cells of the organism, e.g. growth factors, hormones, nutrients. Those survival signals enhance the expression and/or activity of anti-apoptotic regulatory molecules thereby keeping in check the activation of proapoptotic factors **(Ameisen, 2002; Raff, 1993).** Indeed, a set of various antiapoptotic molecules and mechanisms has been identified, as well as proapoptotic factors that counteract those inhibitory molecules when apoptotic demise of a cell is timely and imperative.

1.4.6 The Bcl-2 family

Bcl-2, an oncogene which in follicular lymphoma is frequently linked to an immunoglobulin locus by the chromosome translocation t(14:18), was the first example of an oncogene that inhibits cell death rather than promoting proliferation. B cells transfected with Bcl-2 were shown to be rendered resistant towards apoptosis induced by IL-3 withdrawal: for the first time it was shown that the pathway toward tumorigenesis depends not only on the ability to escape growth control but also depends on the ability to prevent apoptosis **(Vaux, 1988).**

When homologues of Bcl-2 had been identified, it became apparent that a Bcl-2 family of proteins can be defined by the presence of conserved sequence motifs known as Bcl-2 homology domains (BH1 to BH4). In mammals, up to 30 relatives have been decribed of which some belong to a group of pro-survival members and others to a group of proapoptotic members **(Borner, 2003).** In addition to Bcl-2 itself, there are a number of other prosurvival proteins, e.g. Bcl-X_L, Bcl-w, A1, and Mcl-1, which all possess the domains BH1, BH2, BH3, and BH4. The proapoptotic group of Bcl-2 members can be devided into two subgroups: the Bax-subfamily consists of Bax, Bak, and Bok that all possess the domains BH1, BH2, and BH3, whereas the BH3-only proteins (Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, Puma, Blk,

BNIP3, and Spike) have only the short BH3 motif, an interaction domain that is both necessary and sufficient for their killing action **(Cory, 2002; Mund, 2003).**

There has been quite some debate about how the Bcl-2 family controls apoptosis: one model proposes that Bcl-2 members might directly control caspase activation **(Strasser, 2000),** whereas another model claims that they mainly act by guarding mitochondrial integrity **(Wang, 2001).** In support of the first model, the worm Bcl-2 orthologue ced-9 binds to the Apaf-1-like adaptor protein ced-4 and prevents it from activating the caspase ced-3 unless the BH3-only protein Egl-1 displaces ced-4 as shown in **Fig. 3 (Conradt, 1998).** In contrast, the mammalian ced-4 homologue Apaf-1 obviously does not interact with Bcl-2-like proteins **(Moriishi, 1999)** but is activated by cytosolic cytochrome c (see **Fig. 5**), and it is the release of cytochrome c from the mitochondria that can be controlled by Bcl-2 **(Kluck, 1997; Yang, 1997).** Therefore it appears likely, that the central function of mammalian Bcl-2 family members is to guard mitochondrial integrity and to control the release of mitochondrial proteins into the cytoplasm **(Cory, 2002).**

How then is mitochondrial integrity affected by proapoptotic Bcl-2 family members? Central to this question are Bax and Bak, even though inactivation of the Bax gene alone affected apoptosis only slightly and disruption of Bak alone did not show any effect. However, the double knockout of Bax and Bak resulted in dramatic impairment of apoptosis during development in many tissues with superfluous cells accumulating in the hematopoietic system and in the brain. Additionally, cells derived from those Bax -/- Bak -/- mice are insensitive to treatment with e.g. etoposide or irradiation **(Lindsten, 2000; Wei, 2001).** Bax is a cytosolic monomer in viable cells but during apoptosis changes its conformation, integrates into the outer mitochondrial membrane and oligomerizes **(Nechushtan, 2001).** Although the mechanism is controversial, Bax and Bak oligomers are believed to provoke or contribute to the permeabilization of the outer mitochondrial membrane (PT), either by forming channels by themselves **(Antonsson, 200)** or by interacting with components of the PT pore such as VDAC **(Tsujimoto, 2000).**

In contrast, antiapoptotic Bcl-2 members sequester proapoptotic Bcl-2 members by bindig to their BH3 domains and thereby ultimately prevent Bax or Bak activation/ oligomerization and consequently inhibit mitochondrial pro apoptotic events: over expression of Bcl-2 or Bcl-X_L potently inhibits apoptosis in response to many cytotoxic insults, among others by suppressing the generation of ROS, stabilizing $\Delta\psi$, preventing PT and consequently blocking the release of e.g. cytochrome c **(Reed, 1998).** Besides eliciting its anti-apoptotic effects on

the mitochondrial level by indirectly controlling the activation of the apoptosome, Bcl-2 also appears to inhibit apoptotic pathways that are independent of Apaf-1/caspase-9 and which might depend on caspase-7 as a central effector [Marsden, 2002]. In this context one might even expect the existence of another but up to now unidentified Apaf-1 homologue that can be directly controlled by Bcl-2/Bcl-X_L **(Puthalakath, 2002).**

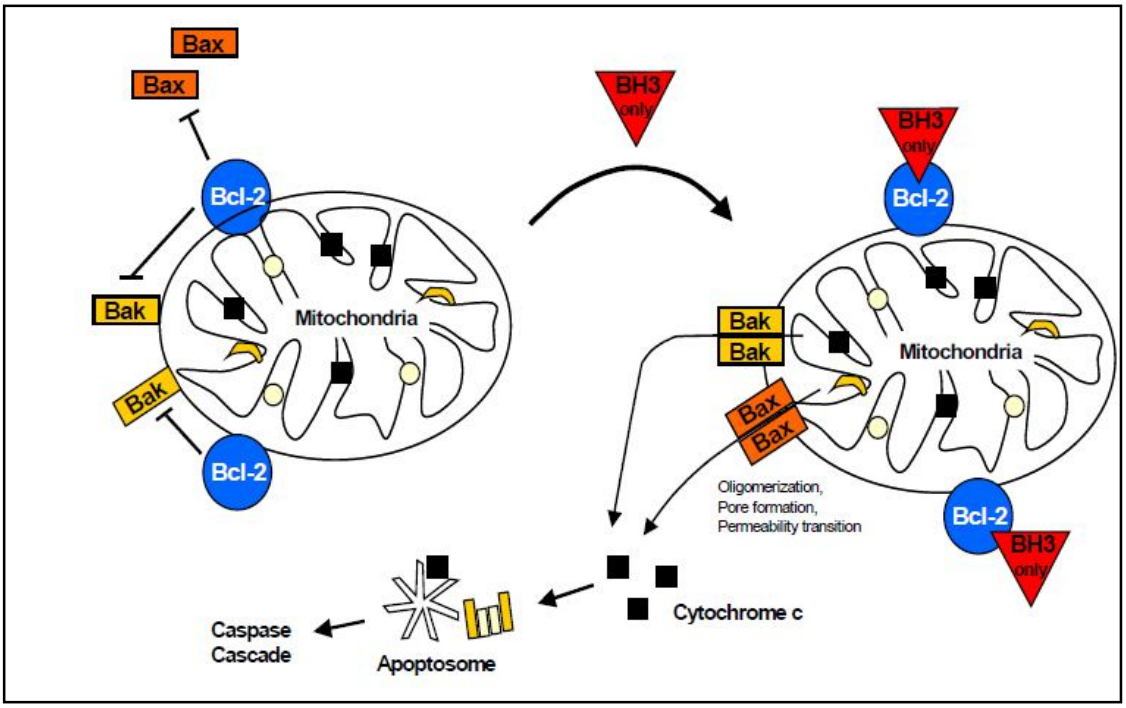


Fig. 7. Regulation of apoptosis by the Bcl-2 family. In a viable cell, the pro-apoptotic Bcl-2 family members Bax, Bak, and BH3-only proteins are antagonized by antiapoptotic members such asBcl-2. In response to an apoptotic stimulus, BH3-only members are activated by transcriptional upregulation (Bax, Noxa, Puma), subcellular relocalization (Bim, Bmf), dephosphorylation (Bad),or proteolysis (Bid). Activated BH3-only proteins prevent antiapoptotic Bcl-2 members from inhibiting proapoptotic members. In addition, they might directly induce a conformational change ofBax and Bak which subsequently oligomerize and insert into the mitochondrial membrane where they form pores either by themselves or by associating with the permeability transition porecomplex. In consequence, proapoptotic factors are released from the inner mitochondrial membrane into the cytosol, such as cytochrome c which contributes to the formation of the apoptosome and the subsequent activation of the caspase cascade.

Whereas Bax and Bak represent the central core of a proapoptotic Bcl-2 death machinery that is held in check by the pro-survival members Bcl-2 and Bcl-X_L, members of the BH3-only subfamily are required for the activation of proapoptotic Bax/Bak function **(Bouillet, 2002).** On the other hand, the killing effect of BH3-only members depends on Bax/Bak, since cells

double-deficient for Bax and Bak do not die upon overexpression of BH3-only proteins as it would be the case in wildtype cells, indicating that BH3-only members function upstream of Bax and Bak **(Lindsten, 2000; Wei, 2001).** Importantly, just as the proapoptotic activity of multidomain proteins Bax and Bak is controlled by their interaction with the antiapoptotic guardians Bcl-2/Bcl-X_L, also most BH3-only members display a strong binding preference to antiapoptotic Bcl-2/Bcl-X_L and in this way are kept under control **(Scorrano, 2003).**

Individual BH3-only proteins are believed to transduce specific death signals since they can be activated for apoptosis signalling by sensing cell stress, such as DNA damage (Noxa and Puma are p53-inducible genes), growth factor deprivation (Hrk and Bim mRNA expression is increased), or anoikis (Bmf is activated by subcellular relocalization) **(Borner, 2003).** As another example, cytoplasmic Bid is processed by caspase-8 to its truncated form tBid, which after myristoylation translocates to the mitochondria where it triggers cytochrome c release by affecting Bax/Bak oligomerization and/or by mobilizing cytochrome c stores in cristae **(Cory, 2002; Scorrano, 2002).**

In general, BH3-only proteins are thought to interfere with the fine-tuned balance of homo- or heterooligomerization between proapoptotic multidomain members Bax/Bak and antiapoptotic members Bcl-2/Bcl-XL (**Fig. 7**). It has been proposed that Bid and Bim possess BH3 domains (Bid-like BH3 domain) which can directly mediate Bax/Bak oligomerization, whereas Bad and Bik possess Bad-like BH3 domains which do not directly act on Bax/Bak but preferentially interact with antiapoptotic Bcl-2/Bcl-XL. As a consequence, activated Bad/Bik might be able to displace Bid/Bim from the binding pocket of antiapoptotic Bcl-2/Bcl-XL, and - in this way released - Bid/Bim might provoke Bax/Bak oligomerization and cytochrome c release even at subliminal levels **(Letai, 2002).**

In summary, a current model of how Bcl-2 family members regulate apoptosis can be descibed as follows (**Fig. 7**): specific apoptotic stress signals trigger the activation of particular BH3-only proteins which then interact with antiapoptotic members on the outer mitochondrial (but also nuclear/ER) membrane, resulting in the release of Bax-like proapoptotic factors. Bax-like factors undergo a conformational change (possibly assisted by some BH3-only proteins), insert into the outer mitochondrial membrane where they provoke PT and the release of apoptogenic factors **(Borner, 2003).**

1.4.7 Regulation of apoptosis by IAPs

Expression levels of antiapoptotic proteins such as Bcl-2, Bcl-X_L, and A1 were reported to be upregulated by the transcription factor NF-κB which besides being a central regulator of the innate and adaptive immune response is commonly described as an antiapoptotic transcription factor **(Heckman, 2002; Karin, 2002),** although under certain circumstances NF-κB also might positively contribute to apoptosis induction [Grimm, 1996]. Besides inducing the expression of pro-survival Bcl-2 members, NF-κB additionally transactivates a number of other antiapoptotic genes, such as the IAPs (inhibitors of apoptosis proteins).

IAPs are a family of antiapoptotic proteins whose prototype originally was described in baculovirus with many homologues found to be conserved across several species. So far, eight human IAP homologues have been identified, among others NAIP, c-IAP1, c-IAP2, XIAP and survivin. All IAPs contain baculovirus IAP repeat (BIR) domains, 70 amino acid motifs, which are essential for the antiapoptotic properties of IAPs **(Takahashi et al., 1998)** because it is the interaction between the BIR domains and caspases that is believed to confer most of the antiapoptotic activity of IAPs. Indeed, XIAP, c-IAP1 and c-IAP2 are thought to directly inhibit caspases-3, -7, and –9 **(Salvesen et al., 2002a)**. In case of XIAP, it is the BIR3 domain that directly binds to the small subunit of caspase-9, whereas it is the BIR2 domain that interacts with the active-site substrate binding pocket of caspases-3 and –7 **(Huang, 2001; Srinivasula, 2001).**

In addition to the BIR domains, c-IAP1, c-IAP2, and XIAP contain a highly conserved RING domain at their C-terminal end which possesses E3 ubiquitin ligase activity. Via this RING domain, IAPs are able to catalyze their own ubiquitination, thereby targeting themselves for degradation by the proteasome **(Yang, 2000),** but they also might target other proteins such as caspase-3 and –7 for ubiquitination and degradation **(Huang, 2000; Suzuki, 2001).** Direct inhibition of caspase activity by c-IAPs is certainly a very important means of regulation when considered that signalling cascades mediated by proteolytic enzymes such as caspases is irreversible once activated and therefore must be precisely regulated in order to prevent locally and temporally inappropriate demise of cells. Importantly, Smac/Diablo, when released from the mitochondrial intermembrane space during mitochondrial apoptotic events, is able to counteract the inhibitory effect of IAPs on caspases since Smac/Diablo can bind to e.g. XIAP in a manner that displaces caspases from XIAP and enables their activation. Thus, Smac/Diablo is a negative regulator of IAPs and in this way unfolds its apoptosis-enhancing property **(Du, 2000).**

1.4.8 Disease as a consequence of dysregulated apoptosis

In the adult human body several hundred thousand cells are produced every second by mitosis, and a similar number die by apoptosis for the maintenance of homeostasis and for specific tasks such as the regulation of immune cell selection and activity **(Fadeel, 1999b).** Dysregulation of apoptotic signaling can play a primary or secondary role in various diseases with insufficient apoptosis leading to e.g. cancer (cell accumulation, resistance to therapy, defective tumor surveillance by the immune system), autoimmunity (failure to eliminate autoreactive lymphocytes), persistent infections (failure to eradicate infected cells), whereas excessive apoptosis contributes to e.g. neurodegeneration (Alzheimers' disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis), autoimmunity (uncontrolled apoptosis induction in specific organs), AIDS (depletion of T lymphocytes), and ischaemia (stroke, myocardial infarction) **(Reed, 2002).** Malfunction of the death machinery results from the mutation of genes that code for factors directly or indirectly involved in the initiation, mediation, or execution of apoptosis, and several mutations in apoptosis genes have been identified as a causing or contributing factor in human diseases **(Mullauer, 2001).**

Of special interest is the involvement of defective apoptosis pathways in tumor formation, progression, and metastasis as well as the occurrence of multidrug resistance during cancer therapy **(Johnstone, 2002)**. During the last years it became more and more evident that tumorigenesis is not merely the result of excessive proliferation due to the activation of oncogenes but to the same extent depends on the – frequently concurrent - impairment of apoptosis checkpoints **(Hanahan, 2000; Wang, 1999).** Intriguingly, many of the alterations that induce malignant transformation, such as oncogene-driven deregulated proliferation and invasion, actually sensitize a cell to apoptosis, and therefore only those oncogenic transformed cells will survive and become malignant which additionally acquire defects in apoptosis pathways and therefore are protected against cell death induction **(Vousden, 2002).** A transformed cell can achieve protection against apoptosis by inappropriate activation or expression of antiapoptotic proteins (which usually act as oncogenes), or by the inactivation of proapoptotic factors (which usually are tumor-suppressors).

As an example and as already mentioned, Bcl-2 was the first apoptosis-related gene that was recognized to play a role in tumorigenesis, and indeed, Bcl-2 is overexpressed in a variety of cancers, contributing to cancer cell survival through direct inhibition of apoptosis **(Hockenbery, 1990; Reed, 1999).** Conversely, mutated or downregulated Bax and Bak are

observed in certain cancers **(Kondo, 2000; Rampino, 1997)** and disruption of those genes promotes tumorigenesis in mice **(Yin, 1997).** Further, proapoptotic Bad and procaspase-9 are negatively regulated by the oncogenic Akt/PKB kinase, which on the other hand is frequently constitutively active or amplified in many types of human cancer **(Nicholson, 2002),** and its antagonist, the phosphatase PTEN, is one of the most commonly mutated tumor-suppressors **(Yamada, 2001)**. Even more underlining its potential as an oncogene, Akt/PKB is also stimulating the NF-κB survival pathway by phosphorylation of IκB kinase α (IKK α) and it is suppressing p53 proapoptotic signalling by phosphorylation of the oncogene Mdm2 which thereby is activated for inhibition of p53 **(Mayo, 2002)**. Both, NF-κB and Mdm2, are themselves inappropriately activated or overexpressed in the process of transformation **(Chene, 2003; Orlowski, 2002).**

A paradigm for the central importance of apoptosis checkpoints in the defense against malignant transformation presents the tumor suppressor p53, which is presumably the most intensely studied apoptosis factor contributing to cancer because it is inactivated in presumably more than 50% of all human cancers **(Hainaut, 2000).** p53 is a tumor suppressor protein which is activated as a transcription factor in response to e.g. oncogene activation, hypoxia and especially DNA damage, resulting in growth arrest and/or apoptosis by stimulating the expression of various p53 target genes such as p21, Bax, Puma, Noxa, Apaf-1, Fas, and DR5 **(Vousden, 2002)** or by repressing the expression of antiapoptotic proteins, e.g. Bcl-2, Bcl-XL or survivin **(Hoffman, 2002; Wu, 2001).** Recent evidence suggests transcription-independent p53 apoptosis pathways in which p53 translocates to the mitochondria, interacts with Bcl-X_L, induces PT and the release of cytochrome c **(Mihara, 2003)**.

In non-stressed, undamaged cells p53 therefore must be kept under stringent control: it is present only at low cellular concentrations, it is retained in the cytosol and prevented to enter the nucleus, and its transactivation domain is inactivated **(Chene, 2003)**. Central to p53 regulation is the oncogene Mdm2 which binds to and thereby inhibits p53 (see **Fig. 8**). Mdm2 is a ubiquitin-ligase which mediates ubiquitination of p53, thereby targeting it for degradation by the proteasome. In this way, p53 levels are kept low in normal cells **(Kubbutat, 1997)**. The importance of Mdm2 in the control of p53 is demonstrated by mdm2 gene knockout mice which die early during development but are rescued from death by additional deletion of the p53 gene **(Montes de Oca Luna, 1995).**

In response to cellular stress (such as DNA damage), p53 is phosphorylated at specific serine/threonine residues which prevents the Mdm2-p53 interaction, and thus p53 is stabilized and activated **(Schon, 2002).** Moreover, p53 is central to oncogene-induced cell death because it is induced by oncogenes such as c-myc, adenovirus E1A, and ras as well as by loss of the retinoblastoma tumor suppressor pRb **(Henriksson, 2001).** All those oncogenes activate the transcription factor E2F-1 which not only can promote cell cycle progression and proliferation but at the same time directly triggers expression of the tumor suppressor ARF which leads to stabilization and activation of p53 **(Ginsberg, 2002).** This explains in part why oncogene activation not always leads to uncontrolled cell proliferation but under certain circumstances to the stabilization of p53 and activation of cell death, provided that p53 signalling pathways are intact **(Eischen, 1999).**

Therefore in many instances, an oncogenic insult only results in increased proliferation and eventually malignant transformation if activators of p53 (such as ARF, Chk2, or ATM), p53 itself (e.g. by Mdm2, the adenovirus E1B, papillomavirus E6, or SV40 large T antigen), or p53 downstream signaling components (p53 target genes) are inactivated (**Fig. 8**).

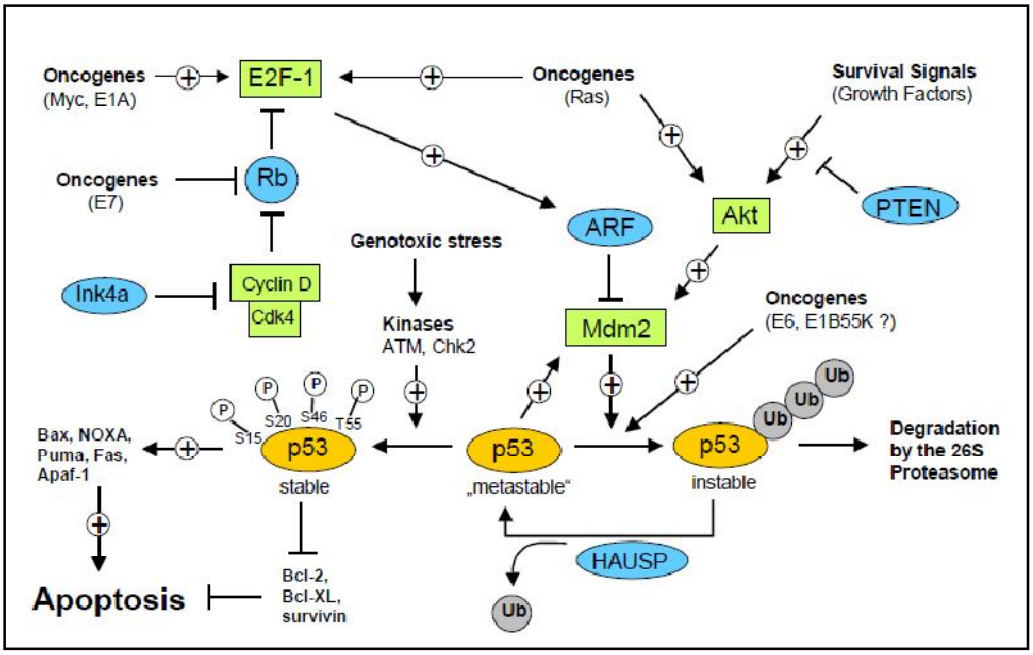


Fig. 8. The p53 network – survival and cell death regulation. In a normal growing viable cell, the p53protein is in a metastable state, i.e. p53 is susceptible to targeted ubiquitination and subsequent proteasomal degradation. Mdm2 directly interacts with p53 and thereby catalyzes ubiquitination ofp53. Ubiquitination of p53 can be reversed by the action of the deubiquitinating enzyme HAUSPwhich thereby can rescue p53 from degradation. p53 is stabilized in response to genotoxic stress, such as DNA damage which leads to the phosphorylation of p53 at several specific serine and threonine residues. Stabilized and activated p53 can translocate into the nucleus where it activates the transcription of proapoptotic genes and suppresses the transcription of antiapoptotic genes what under certain conditions can result in the induction of apoptosis. p53-mediated apoptosis signaling is dependent on the interplay of many regulatory factors, including

protooncogenes as well as tumor-suppressors. Mdm2 activity is positively regulated by the action of the Akt kinase: when Mdm2 is populated by Akt, Mdm2 is able totranslocate from the cytosol to the nucleus where it unfolds its inhibitory effect on p53. Akt kinase, on the other hand, is activated in response to survival signals coming from growth factor receptors. This is therefore an instructive example for the negative regulation of proapoptotic, p53-mediated signals by survival signaling. Whereas Akt kinase positively regulates Mdm2 activity,Mdm2-mediated suppression of p53 is blocked by the action of the ARF tumor suppressor. Bybinding to Mdm2, ARF prevents the interaction between Mdm2 and p53 and therefore stabilizesand activates p53. ARF expression is dependent on the transcription factor E2F-1 which is regulated by the retinoblastoma (Rb) tumor-suppressor and by the action of oncogenes. As anexample, mitogenic signals lead to the activation of oncogenes such as c-myc and ras whichamong others activate E2F-1, resulting in increased ARF activity, stabilization of p53 and induction of apoptosis. Therefore, increased mitogenic signaling or inappropriate oncogenic activity not necessarily causes excessive proliferation but in cells with intact p53 signaling pathways can actas apoptosis inducers.

On the other hand, p53-mediated apoptosis pathways can be suppressed by survival signals, such as growth factors binding to their cognate growth factor receptors what eventually results in activation of the Akt kinase **(Datta, 1999)**. Akt kinase is known to mediate a number of antiapoptotic mechanisms, such as the direct phosphorylation and inactivation of Bad and caspase-9, the activation of NF-κB anti-apoptotic signaling via phosphorylation of IκB, but also phoshorylation and activation of Mdm2 as an inhibitor of p53 **(Mayo, 2002)**. Besides phosphorylation, ubiquitination and protein-protein interactions, p53 is also regulated by acetylation what affects its transcriptional activity, as well as by sumoylation **(Melchior, 2003; Appella, 2001).**

Gaining insight into the mechanisms and alterations by which components of the apoptotic machinery contribute to pathogenic processes, should allow the development of more effective, higher specific and therefore better-tolerable therapeutic approaches. Those may include the targeted activation of proapoptotic tumor suppressors or alternatively the blockade of antiapoptotic oncogenes in the case of cancer, whereas for the treatment of premature cell death during e.g. neurodegeneration the inhibition of proapoptotic key components such as the caspases might be promising **(Reed, 2002).**

Chapter 2

REVIEW OF LITERATURE

Already since the mid-nineteenth century, many observations have indicated that cell death plays a considerable role during physiological processes of multicellular organisms, particularly during embryogenesis and metamorphosis **(Gluecksmann, 1951; Lockshin, 2001).** The term programmed cell death was introduced in 1964, proposing that cell death during development is not of accidental nature, but follows a sequence of controlled steps leading to locally and temporally defined self-destruction **(Lockshin, 1964).**

Eventually, the term apoptosis had been coined in order to describe the morphological processes leading to controlled cellular self-destruction **(Kerr *et al.*, 1972)**. Apoptosis is of Greek origin, having the meaning "falling off or dropping off", in analogy to leaves falling off trees or petals dropping off flowers. This analogy emphasizes that the death of living matter is an integral and necessary part of the life cycle of organisms. The apoptotic mode of cell death is an active and defined process which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions. It should be stressed that apoptosis is a well-defined and possibly the most frequent form of programmed cell death, but that other, non-apoptotic types of cell death also might be of biological significance **(Leist, 2001).**

2.2 Apoptosis as a Novel Target for Cancer Chemoprevention

Cancer chemopreventive agents are typically natural products or their synthetic analogs that inhibit the transformation of normal cells to premalignant cells or the progression of premalignant cells to malignant cells. These agents are believed to function by modulating processes associated with xenobiotic biotransformation, with the protection of cellular elements from oxidative damage, or with the promotion of more differentiated phenotype in target cells. However, an increasing number of chemopreventive agents (e.g., certain retinoids, nonsteroidal anti-inflammatory drugs, polyphenols, and vanilloids) have been shown to stimulate apoptosis in premalignant and malignant cells *in vitro* or *in vivo***. (Sun *et al.*, 2004).**

Anticancer drugs act through different pathways converging ultimately into activation of apoptosis in cancer cells leading to cell cytotoxicity. Recent studies have documented that two major pathways are involved in the regulation of apoptosis. The drugs may kill cells either by activation of extrinsic or intrinsic apoptotic pathways. The extrinsic apoptotic pathway involves cell surface death receptors, such as Fas/CD95 and TNFR1, which upon activation up regulate downstream signalling cascade leading to the activation of caspase-8 **(Wilson, 1998)**. The intrinsic pathway is dependent on various cell stress stimuli leading to altered ratio of Bcl-2 family members affecting cytochrome c and apoptotic protease activating factor-1 (Apaf-1) release that leads to caspase-9 activation **(Sun *et al.*, 1999).**Since the mitochondrial respiratory chain (electron transport complexes) is a major source of ROS generation in the cells, the vulnerability of the mitochondrial DNA to ROS-mediated damage appears to be a mechanism to amplify ROS stress in cancer cells. The escalated ROS generation in cancer cells serves as an endogenous source of DNA-damaging agents that promote genetic instability **(Ghafourifar *et al.*, 2001).**

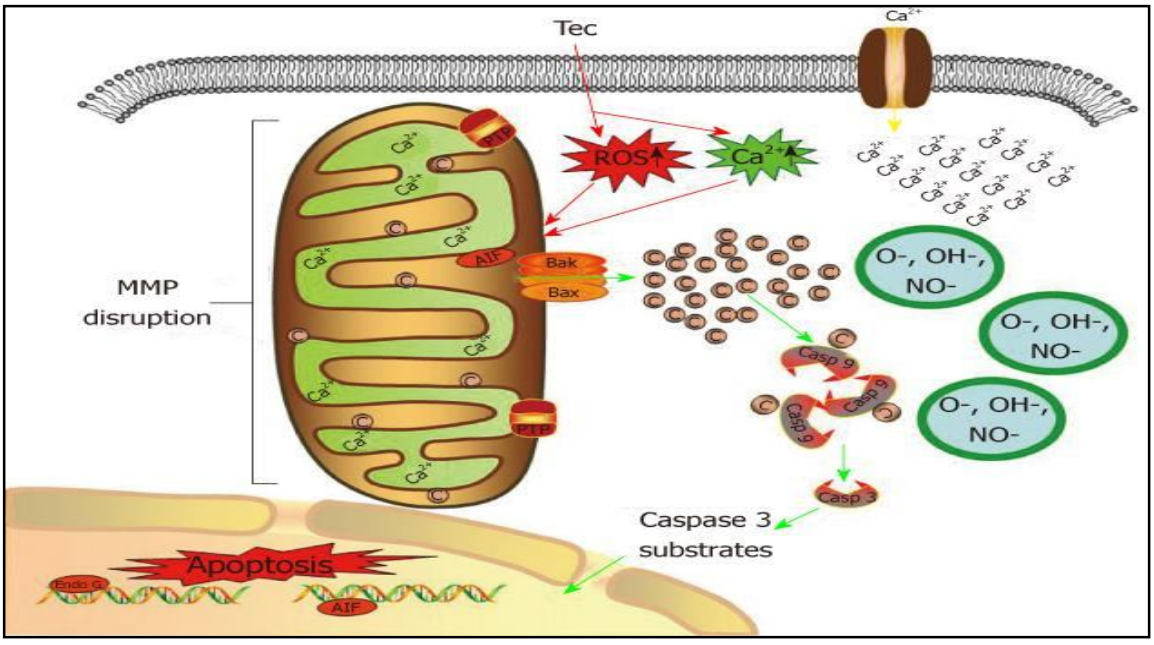


Figure 1: ROS generation & mitochondrial dysfunction in cell death **(Malik *et al.*, 2007).**

Inhibition of apoptosis leads to activation of cell survival factors (e.g., AKT) causes continuous cell proliferation in cancer. Apoptosis, the major form of cellular suicide, is

central to various physiological processes and the maintenance of homeostasis in multicellular organisms. A number of discoveries have clarified the molecular mechanism of apoptosis, thus clarifying the link between apoptosis and cell survival factors, which has a therapeutic outcome. Induction of apoptosis and inhibition of cell survival by anticancer agents has been shown to correlate with tumor response. Cellular damage induces growth arrest and tumor suppression by inducing apoptosis, necrosis and senescence; the mechanism of cell death depends on the magnitude of DNA damage following exposure to various anticancer agents. Apoptosis is mainly regulated by cell survival and proliferating signaling molecules. As a new therapeutic strategy, alternative types of cell death might be exploited to control and eradicate cancer cells **(Kumar *et al.*, 2013).**

Drug	**Mechanism of action**	**Plant source**
Vinblastine, vincristine	Inhibition of tubulin polymerization	*Catharanthus roseus* (Apocynaceae)
Etoposide, teniposide	Inhibition of topoisomerase II	*Podophyllum peltatum*, *P. emodi* (Berberidaceae)
Paclitaxel, docetaxel	Promotion of tubulin stabilization	*Taxus brevifolia* (Taxaceae)
Irinotecan, topotecan, 9-aminocampothecin, 9-nitrocamptothecin	Inhibition of topoisomerase I	*Camptotheca acuminata* (Nyssaceae)
Homoharringtonine	Inhibition of DNA polymerase	*Harringtonia cephalotaxus* (Cephalotaxaceae)
4-Ipomeanol	Cytochrome P-450-mediated conversion into DNA-binding metabolites	*Ipomoeca batatas* (Convolvulaceae)
Elliptinium	Inhibition of topoisomerase II	*Bleekeria vitensis* (Apocynaceae)
Flavopiridol	Inhibition of cyclin-dependent kinases	*Amoora rohituka*; *Dysoxylum binectariferum* (Maliaceae)

Table 1: Some Anti-cancer drugs developed from plant sources.

2.3 NATURAL PRODUCTS AS ANTICANCER AGENTS

Cancer chemoprevention can be achieved by the use of natural, synthetic or biologic compounds that reverse, suppress or prevent the development of epithelial malignancies. Natural compounds including flavonoids are able to reduce oxidative stress, which is the most likely mechanism mediating the protective effects against cancer development. In addition, *in vitro* and *in vivo* studies have suggested that flavonoids, such as (-)-epigallocatechin-3-gallete (EGCG), quercetin, and curcumin, act by induction of apoptosis. Certain natural products have been shown to inhibit the activation of nuclear factor kappa B (NF-κB) and Akt signaling pathways, both of which are known to maintain a homeostatic balance be- tween cell survival and apoptosis **(Kuno et al.,2012)**.

2.3.1Plant derived anti-cancer drugs

Very few drugs currently being used for the treatment of cancer were discovered on the basis of rationale structural design. One notable exception is 5-fluorouracil, conceptualized and evaluated by Heidelberger and coworkers **(Heidelberger *et al.*, 1957).** Most other agents have been discovered as a result of empiricism, serendipity, or large-scale evaluation (screening) programs. Drug discovery from medicinal plants has played an important role in the treatment of cancer and, indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied towards combating cancer **(Butler, 2004).** From all available anticancer drugs between 1940 and 2002, 40% were natural products per se or natural product-derived with another 8%considered natural product mimics **(Newman et al., 2003).** Some of the plant-derived compounds were also of great significance to cancer therapy (Table 1).

2.3.2 Phytochemicals as chemotherapeutics against cancer cells

Most of the phytochemicals have been recognized to have potential chemopreventive or chemotherapeutic efficacy in cancer treatment. Berberine, a naturally occurring isoquinoline alkaloid, has been shown to possess anti-inflammatory and antitumor properties in some in vitro systems. In this, in vitro treatment of androgen-insensitive (DU145 and PC-3) and androgen-sensitive (LNCaP) prostate cancer cells with berberine inhibited cell proliferation and induced cell death in a dose-dependent (10–100 Mmol/L) and time-dependent (24–72 hours) manner (Mantenna et al., 2006). It has been documented that berberine would have anticancer activities in SCC-4 human tongue cancer cells. Results indicated that berberine reduced the viability of SCC-4 cells, which was initiated by the generation of reactive oxygen species, via an increase in cytosolic Ca2+. Berberine-induced apoptosis was associated with a

reduction of the mitochondrial membrane potential associated with changes in the Bax/Bcl-2 ratio, release of cytochrome c from mitochondria and activation of downstream caspase-3. Real-time PCR showed that berberine stimulated gene expression of caspase-8, -9 and - 3, apoptosis-inducing factor and endonuclease G. The present study demonstrated that berberine-mediated apoptosis of SCC-4 cells is regulated by ROS, mitochondria, caspase-3-dependent and mitochondria-dependent pathways, suggesting that berberine may be considered for future studies as a promising therapeutic candidate for human tongue cancer (Ho *et al.*, 2009).

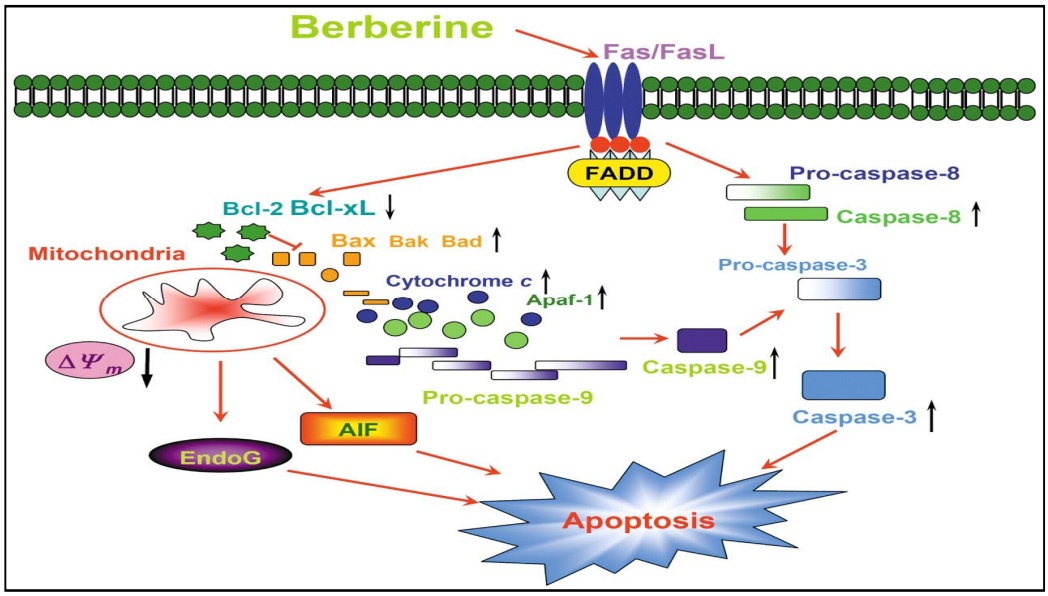


Figure 2: Berberine-mediated apoptosis in SCC-4 human tongue cancer cells (Yang et al., 2009).

2.4 CHALCONE DERIVATIVES: NOVEL TARGET AGAINST CANCER

Chalcones (1,3-diphenyl-2-propen-1-ones)—one of the major classes of natural products with widespread distribution in spices, tea, beer, fruits and vegetables—have been recently subject of great interest for their pharmacological activities. Chalcones are precursor compounds in flavonoid biosynthesis in plants. Chemically they consist of open-chain flavonoids in which the two aromatic rings are joined by a three-carbon *α,β*-unsaturated carbonyl system. Chalcones have been reported to possess anti-inflammatory, antimicrobial, antioxidant and anticancer properties **(Ewelina Szliszka et al., 2009).**

Chalcones are contained in fruits and vegetables, and have been suggested to display anticancer activities. Methoxychalcone derivative induced time- and concentration-

dependent G1 arrest of the cell cycle and subsequent apoptosis in human prostate cancer cells. The G1-arrest effect was confirmed by down-regulated expressions of several G1-phase regulators, including cyclin D1, cyclin E, cyclin-dependent kinase (Cdk)-4, Cdk2, phospho-RB, E2F-1, and Cdc25A. The mRNA expressions of cyclin D1 and cyclin E were also inhibited through the suppression of NF-kappaB. Methoxychalcone derivative blocked the protein synthesis and inhibited mammalian target of rapamycin (mTOR) signaling pathways. The suppression of mTOR pathways were irrespective of Akt- and AMPK-activated protein kinase (AMPK), but were attributed to mitochondrial stress, in which the down-regulation of survivin protein level may play a crucial role **(Sun YW et al.,2010).**.Effects of Methoxy- and fluoro-chalcone derivatives were investigated especially on the proliferation of human melanoma cells and peripheral blood mononuclear cells (PBMCs). Four out of the 12 synthetic chalcones: 4-trifluoromethyl-4'-methoxychalcone (CH-1), 4-trifluoromethyl-2'-methoxychalcone (CH-3), 3-trifluoromethyl-2',4'-dimethoxychalcone (CH-4) and 3-trifluoromethyl-4'-methoxychalcone (CH-7) exhibited significant antiproliferative efficacies against the cultured cells of the human melanoma cell line A375. CH-1, CH-3, CH-4, and CH-7 induced cell cycle arrest at uthe S-G(2)/M phase within 24 h after the treatment. CH-3, CH-4, and CH-7 significantly activated caspase-3 at 12 h, subsequently induced apoptosis at 72 h. All chalcones inhibited concanavalin A-induced proliferation of PBMCs dose-dependently. Our results suggest that some methoxy- and/or fluoro-chalcones have antitumor efficacy by inducing apoptosis and the cell-cycle arrest **(Henmi K et al.,2009).** A new chalcone derivative (E)-3-(4-methoxyphenyl)-2-methyl-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one, (CHO27) with an up to 1000-fold increased cytotoxic potency relative to its parent compound in cell culture assays. CHO27 at low nanomolar levels, inhibited prostate cancer (PCa) cell growth through cell cycle arrest and caspase-dependent apoptosis. Activation of p53 accounted for, at least in part, the growth inhibition by CHO27 in vitro. Furthermore, i.p. administration of CHO27 suppressed the growth of established PCa 22Rv1 xenograft tumors accompanied with p53 and p21 (Cip1) induction. CHO27 may be a lead for development of new therapeutic agents for PCa **(Zhang Y et al.,2012).**

Compounds derived from thiophene chalcones (6-17) exhibited generally better antiproliferative activity than those derived from bioisoteric replacement of furan chalcones (18-29) on MDA-MB231 breast cancer cells. In contrast, the compounds derived from furan chalcones showed generally better antiproliferative activity on MDA-MB468 breast cancer cells. Among 24 compounds examined, compounds 21 and 23 showed significantly improved antiproliferative activity against MDA-MB231 and MDA-MB468 cancer cells. However,

compound 23 ((E)-1-(4-chlorophenyl)-3-(5-(4-methoxyphenyl)furan-2-yl)prop-2-en-1-one) is considered to be most desirable among this series, since its antiproliferative activity was 3 to 7-fold higher on cancer than non-cancer cells. Compound 23 showed not only more effective activity than the widely prescribed cisplatin on cancer cells, but it also showed differential antiproliferative activity against cancer cells, a property that is not shown with cisplatin. If this property shown in cell culture stands in vivo test, compound 23 can be an effective and safe anticancer drug **(Solomon VR et al., 2012).**

Imidazo[2,1-b]thiazole chalcone derivatives were synthesized and evaluated for their anticancer activity. These chalcone derivatives show promising activity, with log GI (50) values ranging from -7.51 to -4.00. The detailed biological aspects of these derivatives toward the MCF-7 cell line were studied. These chalcone derivatives induced G(0)/G(1)-phase cell-cycle arrest, down-regulation of G(1)-phase cell-cycle regulatory proteins such as cyclin D1 and cyclin E1, and up-regulation of CDK4. Moreover, these compounds elicit the characteristic features of apoptosis such as enhancement in the levels of p53, p21, and p27, suppression of NF-κB, and up-regulation of caspase-9. One of these chalcone derivatives, 3 d, is potentially well suited for detailed biological studies, either alone or in combination with existing therapies **(Kamal A et al., 2010).** Involvement of pro-apoptotic protein (Bax), active caspase-9 and cleavage of retinoblastoma protein was studied. Interestingly, the chalcone- imadazolone caused upregulation of p21, check point proteins (Chk1, Chk2) and as well as their phosphorylated forms which are known to regulate the DNA damage pathway. Increased p53BP1 foci by immunolocalisation studies and TRF1 suggested the possible involvement of telomere and associated proteins in the apoptotic event. The telomeric protein such as TRF2 which is an important target for anticancer therapy against human breast cancer was extensively studied along with proteins involved in proper functioning of telomeres **(M Janaki Ramaiah et al., 2011).** Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a naturally occurring anticancer agent that induces apoptosis in cancer cells and is not toxic to normal cells. We examined the cytotoxic and apoptotic effect of five chalcones in combination with TRAIL on prostate cancer cells. The cytotoxicity was evaluated by the MTT and LDH assays. The apoptosis was determined using flow cytometry with annexin V-FITC. Our study showed that all five tested chalcones: chalcone, licochalcone-A, isobavachalcone, xanthohumol, butein markedly augmented TRAIL-mediated apoptosis and cytotoxicity in prostate cancer cells and confirmed the significant role of chalcones in chemoprevention of prostate cancer **(Ewelina Szliszka et al., 2009).**

Flavokawain A is the predominant chalcone from kava extract. We have assessed the mechanisms of flavokawain A's action on cell cycle regulation. In a p53 wild-type, low-grade, and papillary bladder cancer cell line (RT4), flavokawain A increased p21/WAF1 and p27/KIP1, which resulted in a decrease in cyclin-dependent kinase-2 (CDK2) kinase activity and subsequent G(1) arrest. The increase of p21/WAF1 protein corresponded to an increased mRNA level, whereas p27/KIP1 accumulation was associated with the down-regulation of SKP2, which then increased the stability of the p27/KIP1 protein. The accumulation of p21/WAF1 and p27/KIP1 was independent of cell cycle position and thus not a result of the cell cycle arrest. In contrast, flavokawain A induced a G(2)-M arrest in six p53 mutant-type, high-grade bladder cancer cell lines (T24, UMUC3, TCCSUP, 5637, HT1376, and HT1197). Flavokawain A significantly reduced the expression of CDK1-inhibitory kinases, Myt1 and Wee1, and caused cyclin B1 protein accumulation leading to CDK1 activation in T24 cells. Suppression of p53 expression by small interfering RNA in RT4 cells restored Cdc25C expression and down-regulated p21/WAF1 expression, which allowed Cdc25C and CDK1 activation, which then led to a G(2)-M arrest and an enhanced growth-inhibitory effect by flavokawain A. Consistently, flavokawain A also caused a pronounced CDK1 activation and G(2)-M arrest in p53 knockout but not in p53 wild-type HCT116 cells. This selectivity of flavokawain A for inducing a G(2)-M arrest in p53-defective cells deserves further investigation as a new mechanism for the prevention and treatment of bladder cancer **(Tang Y et al.,2008).**

Effects of synthetic chalcone derivatives were investigated especially on the proliferation of human melanoma cellsand peripheral blood mononuclear cells (PBMCs). Four out of the 12 synthetic chalcones: 4-trifluoromethyl-4_-methoxychalcone (CH-1), 4-trifluoromethyl-2_-methoxychalcone(CH-3) 3-trifluoromethyl-2_,4_dimethoxychalcone(CH-4) and 3-trifluoromethyl-4_-methoxychalcone (CH-7) exhibited significant antiproliferative efficacies against the cultured cells of the human melanoma cell line A375. CH-1, CH-3, CH-4, and CH-7 induced cell cyclearrest at the S-G2/M phase within 24 h after the treatment. CH-3, CH-4, and CH-7 significantly activated caspase-3 at 12 h, subsequently induced apoptosis at 72 h. All chalcones inhibited concanavalin A-induced proliferationof PBMCs dose-dependently. Our results suggest that some methoxy- and/or fluoro-chalcones have antitumorefficacy by inducing apoptosis and the cell-cycle arrest **(Kayo HENMI et al., 2009).**

Chapter 3

MATERIALS AND METHODS

3.1 CHEMICALS AND ANTIBODIES

3-(4,5,-Dimethylthiazole-2-yl) -2,5-diphenyltetrazoliumbromide (MTT), propidium iodide (PI), DNase-free RNase, bovine serum albumin, Rhodamine-123 (Rh123), proteinase-K, penicillin, kanamycin and phenyl methanesulfonyl fluoride (PMSF) were purchased from M/s Sigma Chemicals Co., India. Fetal bovine serum was obtained from M/s GIBCO Invitrogen Corporation, USA. APO-Alert caspases assay kits were purchased from M/s B.D. Clontech. Anti-human antibodies to Bcl-2, Bax, PARP-1, caspase-3, cas pase-8, caspase-9, actin, goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP was purchased from M/s Santa Cruz, USA. Antibodies p110α,p110β,p110γ,DR5,pSTAT3 were purchased from M/s Cell signalling technology, USA. Electrophoresis reagents and protein markers were purchased from M/s Bio-Rad, USA while ECL reagents and Hyperfilm were purchased from M/s Amersham Biosciences, UK. Other reagents used were AR grade and available locally.

Growth media

Incomplete Growth medium: The contents of the vials of RPMI-1640, DMEM, MEM, MCACOY5A medium with 2 mm L-glutamine were dissolved in double distilled water as per supplier's instructions. Kanamycin (100 mg/liter) and Sodium bicarbonate (2 gm/liter) were also added to the medium. The pH of the medium was adjusted to 7.2 and it was sterilized by filtering through 0.2μ filters in laminar flow under sterile conditions. The media were stored in a refrigerator (2-8^{0}C).

Complete growth medium: The growth medium was supplemented with 10% FCS and Penicillin (100 IU/ml, before use) to make it complete growth medium.

Freezing medium

For Adherent cell lines:

Freezing Medium for cryopreservation contained 20% FCS and 10% DMSO (cell culture grade) in growth medium.

For suspension cell lines:

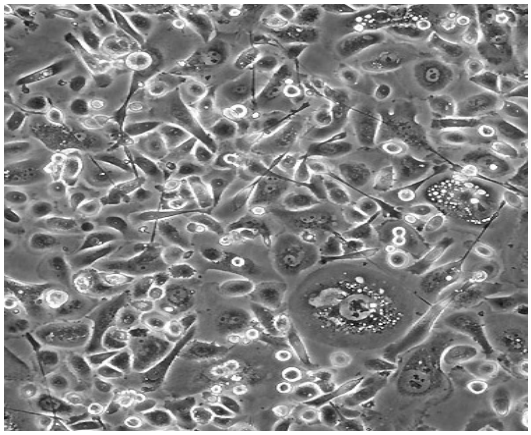
Freezing Medium for cryopreservation contained 95 % FCS and 5 % DMSO (cell culture grade) in growth medium.

Cell culture and treatment

Human colon cancer cell HCT-116, prostate cancer cell line PC-3, pancreatic cancer cell line Miapacca-2, breast cancer cell line MCF-7, lung cancer cell line A549, and promy-elocytic leukaemia cell line HL-60 were obtained from ECACC, UK. The cells were grown in McCoy's 5A/MEM/DMEM/RPMI-1640 medium containing 10% FCS, 100 µg/ml kana-mycin and streptomycin. Cells were grown in a CO2 incubator (Thermocon Electron Corporation, USA) at 37^0C with 5% CO2 gas environment and 95% humidity. Cells grown in monolayer cultures were trypsinised with trypsin (0.1% w/v)/EDTA (1 mM) solution. Soon after cells were ready to detach, the trypsin/EDTA solution was removed. Cells were dispersed gently by pipetting in complete growth medium, centrifuged at 200xg, for 5 min. Cells were dispersed in complete medium in culture flasks and incubated in CO2 incubator. Cells grown in semi-confluent stage (approx. 70% confluent) were treated with test materials dissolved in DMSO while the untreated control cultures received only the vehicle (DMSO, < 0.2%).

3.2 SOURCES OF CELL LINES

The original stock of Human cancer cell lines was received in a frozen state (Dry ice) in cryovials, from the National Cancer Institute (NCI), Fredrick (USA) and some cell lines were obtained from the National Center for Cell Science (NCCS), Pune (India) in culture flasks.



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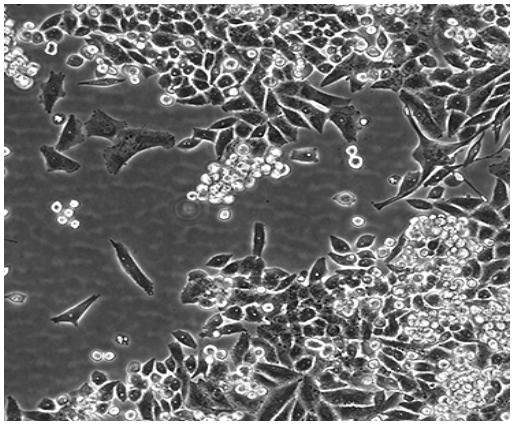

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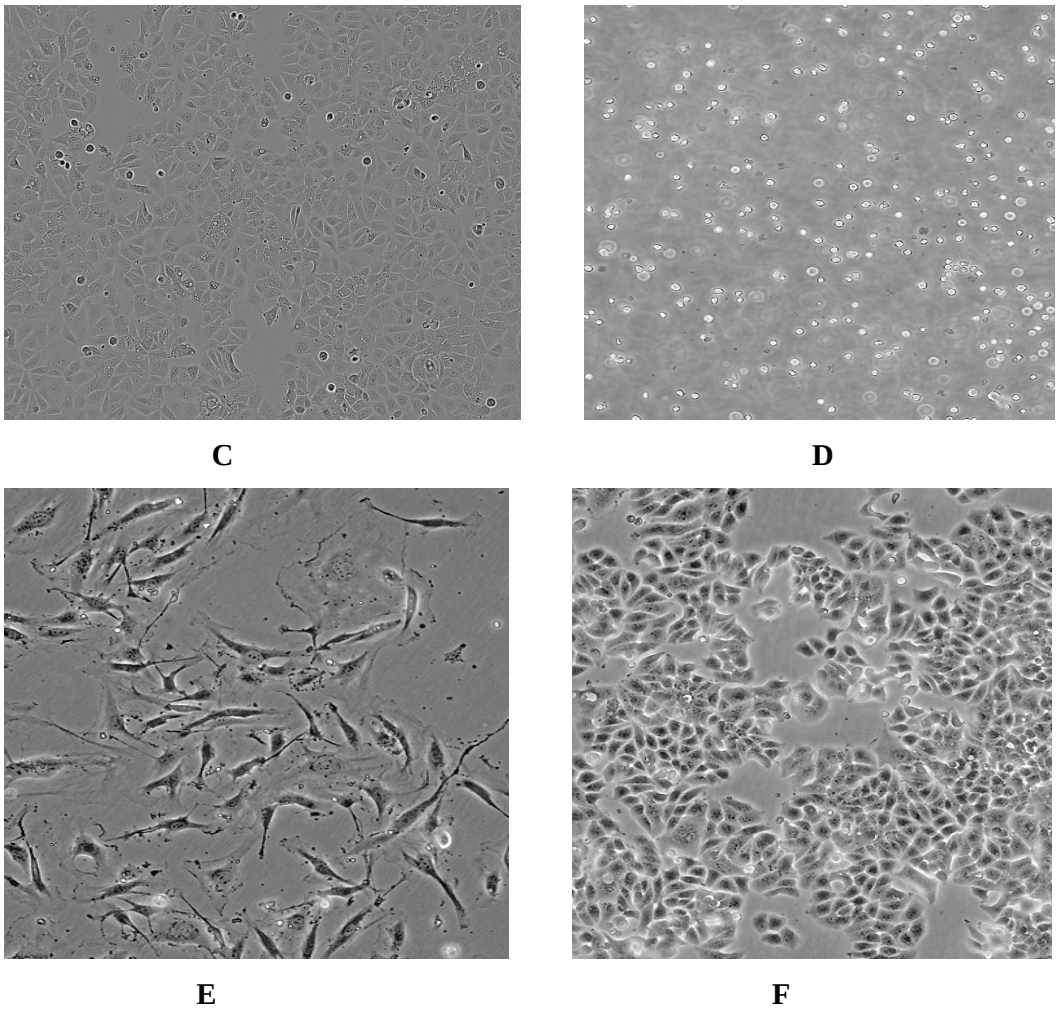

Figure: 11 Represents the morphology of various cell lines of different tissues.
A-PC3 ,-B-MIAPAKA,C-MCF7, D- HL-60,E-HUVEC,F-T47D

Handling of cell line on arrival

Calls in Cryovials received from NCI, USA were transferred to liquid nitrogen immediately on arrival and were used as per requirement. When the cells received in Tissue Culture Flask from NCCS, Pune was observed under aseptic condition. If the flask was found to contain healthy cells without contamination, the contents of the flask were transferred to centrifuge tube and cells with required quantity of the medium were placed in tissue culture flask and grown as described in the latter part of this section.

Revival of cell lines

Cryovials containing cells were removed from the liquid nitrogen container and thawed quickly by shaking in water bath at 37^0C. Cryovials were wiped with 70% alcohol to avoid contamination and transferred to laminar flow. The contents of the vial were transferred into

a sterile centrifuge tube containing 10 ml complete growth medium and centrifuged. Supernatant was discarded and the cells were suspended in fresh complete medium. Cells were mixed properly to ensure uniform distribution in the medium. Cells were transferred to the Tissue Culture Flask -25 containing 7 ml of complete growth medium aseptically and cells were incubated in a CO2 incubator at 37^0C, 5% CO_2 atmosphere and 90% RH.

Cell culture and maintenance of adherent cell lines

Human cancer cells were grown in tissue culture flasks with the complete growth medium at 37ºC in an atmosphere of 5% CO_2 and 90% RH in a carbon dioxide incubator. Cells were daily checked for their proper growth. The medium of the cells was changed when the color became yellow. To change the medium, the medium in the flask was aspirated with pipette-man and discarded. The fresh medium (15-20 ml) was placed in the culture flask under sterile conditions. The flask was properly marked and incubated in CO_2 incubator. Depending on the mass doubling time of cells, sub-culturing of cells was done, when they were at sub-confluent stage.

Cell culture and maintenance of suspension cell lines

Human cancer cells were grown in tissue culture flasks with the complete growth medium at 37ºC in an atmosphere of 5% CO_2 and 90% RH in a carbon dioxide incubator. Cells were daily checked for their proper growth. The medium of the cells was changed when the color became yellow. To change the medium, the medium in the flask was aspirated with pipette-man and kept in a centrifuge tube. Then centrifuge at 1200 rpm for 8-10 minute. The supernatant was discarded and the cell pellet was resuspended with fresh complete growth medium of about 5ml. Then the 5ml cell suspension was transferred to a fresh culture flask containing fresh medium (15-20 ml) placed under sterile conditions. The flask was properly marked and incubated in CO_2 incubator. Depending on the mass doubling time of cells, sub-culturing of cells was done, when they were at sub-confluent stage.

3.3 COUNTING OF THE CELLS

Small volume of media containing cells is placed on the hemocytometer, it is covered with a cover glass, and capillary action completely fills the chamber with the sample. Looking at the chamber through a microscope, the number of cells in the chamber can be determined by counting. Different kinds of cells can be counted separately as long as they are visually distinguishable. The number of cells in the chamber is used to calculate the concentration or density of the cells in the mixture from which the sample was taken.

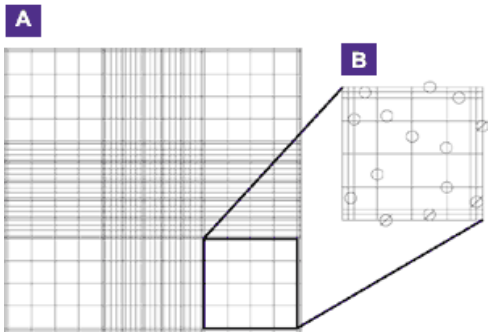

Figure 1: Cell Counting Using Hemocytometer (Manford et al., 1979)

Hemocytometer Counting

- Shake cell suspension well and transfer 20 µl of it to the edge of the chamber. Allow it to be drawn into the chamber by capillary action.
- Count cells in four marginal chambers using 10X objective .
- To calculate :

$C = n/ V$

Where C = cell concentration (cells / ml)

n = average no. of cells counted

v = volume counted = 10^4

Thus conc. of cells/ ml = average no. of cells x dilution factor x 10^4

- After calculating the cell conc. for seeding

$$\frac{\text{Conc. of cells required}}{\text{Conc. of cell / ml}} \times \text{final volume}$$

- Add appropriate volume of cell concentration required to a new flask containing medium (final volume).

Phase Contrast Microscopy

Morphological changes in cell were studied by phase contrast microscopy. Cells were incubated in 6 well plate and treated with different concentration of NPC-007 (.5,1 and 3µM) for 24 h. cells were subjected to phography on an inverted microscope attached to the DP_12 camera.

3.4 CELL PROLIFERATION ASSAY

The cell proliferation assay was performed by using MTT (Bhushan et al.,2007). Human colorectal carcinoma HL-60 cells ($6x10^3$/200µl) were seeded in 96-well culture plates and treated with various concentrations of NPC-007 for 6,12,24 and 48 h. MTT dye was added 4 h prior to experiment termination (250 µg/ml). The MTT formazan crystals formed were dissolved in 150 µl of DMSO and OD was measured at 570 nm (reference wavelength 620 nm). Cell growth as percentage viability was calculated by comparing the absorbance of treated verses untreated cells.

MTT Assay

The MTT assay is a laboratory test and standard colorimetric assays (an assay which measures changes in color) for measuring the activity of enzymes that reduce MTT to formazan, giving a purple color. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials, since those agents would result in cell toxicity and therefore metabolic dysfunction and therefore decreased performance in the assay. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in living cells.[221]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 colored solution. The absorbance of this colored 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.

This assay is a quantitative colorimetric method for determination of cell survival and proliferation. The assessed parameter is the metabolic activity of viable cells. Metabolically active cells reduce pale yellow tetrazolium salt (MTT) to a dark blue water-insoluble formazan which can be, after solubilisation with DMSO, directly quantified. The absorbance of the formazan directly correlates with the number of viable cells.

3.5 INVITRO MECHANISTIC STUDIES

Induction of apoptosis in cancer cells has been a novel approach for innovative mechanism-based anticancer drug discovery. It is thus important to screen apoptotic inducers from plants, either in the form of crude extracts or as components isolated from them. Apoptosis is a cell death process characterized by morphological and biochemical features occurring at different

stages. Once triggered apoptosis proceeds with different kinetics depending on cell types and culminates with cell disruption and formation of apoptotic bodies. To know apoptosis in cancer cells, mechanistic mulitiparamartic assay were done such as detection of morphological changes in cancer cell lines caused by tested compounds through, Hoechst 33258 nuclear staining, Cell cycle analysis, changes in mitochondrial membrane potential through Flowcytometery, Western blotting.

Cell line

Suspension cell line Human leukemia cancer cell HL-60 was used in the mechanistic studies.

Cell culture method for suspension cell lines

Human cancer suspension cells were seeded and grown in tissue culture flasks in complete growth medium (RPMI) at 37ºC in an atmosphere of 5% CO_2 and 90% RH in a carbon dioxide incubator. Cells were checked daily for proper growth and maintains. Change the medium of the flask when the color became phenol red or yellow with the fresh complete growth medium (prewarmed at 37^0C) took out the medium from the flask to 50ml centrifuge tube aseptically and centrifuged at 1000rpm for 10 minutes, discarded the supernatant and resuspended the pellet in fresh RPMI medium and again put into new TCF-75 flask for continuation and a portion of the cells were also cryopreserved. The cell cultures was also maintained for further storage and experiments and marked the flask properly and incubate at 37^oC & 5% CO_2 atmosphere in an incubator.

Cryopreservation

In order to minimize genetic drift in cell lines, to avoid senescence or transformation in infinite cell lines and to guard against accidental loss by contamination or otherwise, it is common a practice to freeze (cryopreserve) aliquots of cells in liquid nitrogen vapors. It is important to bring the cells at -80°C with the cooling rate of 1°C/min at the time of cryopreservation.

The cells of the flask at log phase can be cryopreserved. The cell suspension was taken in 50 ml centrifuge tube and centrifuged at low speed (800rpm). The pellet was suspended in freezing medium containing 95% FCS, 5% DMSO and the cell density was adjusted to $1x10^8$ cells/ml. Cells with more than 98% viability as determined by trypan blue exclusion technique were cryopreserved. The aliquots of 1.0 ml were transferred into cryovials. The temperature of the vials was brought down to -80ºC by using a specially designed box (Mr.Frosty, Sigma Chem Co, USA Cat no. C-1562) which are commercially

available. The cryobox was put into -80ºC refrigerator for 24 hours. Later, the vials were transferred into liquid nitrogen container.

Preparation of cell suspension

When cells are at log phase, healthy and no sign of contamination can be used for mechanistic studies. Medium of the cells was changed one day in advance. It involves centrifugation of cells and resuspended the pellet in fresh medium. An aliquot was taken out; cells were counted and checked for viability with trypan blue. Cell stock with more than 98% cell viability was accepted for determination of *in vitro* cytotoxicity. The cell density was adjusted as per requirement from $1x10^6$ -$1x10^7$cells/ml by the addition of more complete growth medium and inoculated 6 well tissue culture plate and incubated in CO_2 incubator for 24 hrs.

3.6 FLOW CYTOMETERY BASED ASSAY

In a Flow cytometery system, large number of cells or particles flow with in a laminar fluid stream in a single file passing a lasser beam where they are individually evaluated. As the focused laser beam interacts with a cell, scattered light, and in the case using fluorescent antibodies or dyes, fluorescence signals are created at the same time. The electronic signal are converted into digital values and are illustrated in dot plot or histogram plot. All measurements were performed on FACS-BD-LSR™ (Becton Dickinson USA), equipped with 488 nm argon-ion laser, using cell quest software.

Figure: 2 (A&B) Flowcyometer BD FACSAria II at IIIM, Cancer research Lab.

Cell cycle analysis:

The cell cycle, or cell-division cycle, is the series of events that take place in a cell leading to its division and duplication (replication). In cells without a nucleus (prokaryotes), the cell cycle occurs via a process termed binary fission. In cells with a nucleus (eukaryotes), the cell

cycle can be divided in two brief periods: interphase—during which the cell grows, accumulating nutrients needed for mitosis and duplicating its DNA—and the mitosis (M) phase, during which the cell splits itself into two distinct cells, often called "daughter cells". The cell-division cycle is a vital process by which a single-celled fertilized egg develops into a mature organism, as well as the process by which hair, skin, blood cells, and some internal organs are renewed.

The cell cycle consists of five distinct phases: G1 phase, S phase (synthesis), G2 phase (collectively known as interphase) and M phase (mitosis). M phase is itself composed of two tightly coupled processes: mitosis, in which the cell's chromosomes are divided between the two daughter cells, and cytokines, in which the cell's cytoplasm divides forming distinct cells. Activation of each phase is dependent on the proper progression and completion of the previous one. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G0 phase.

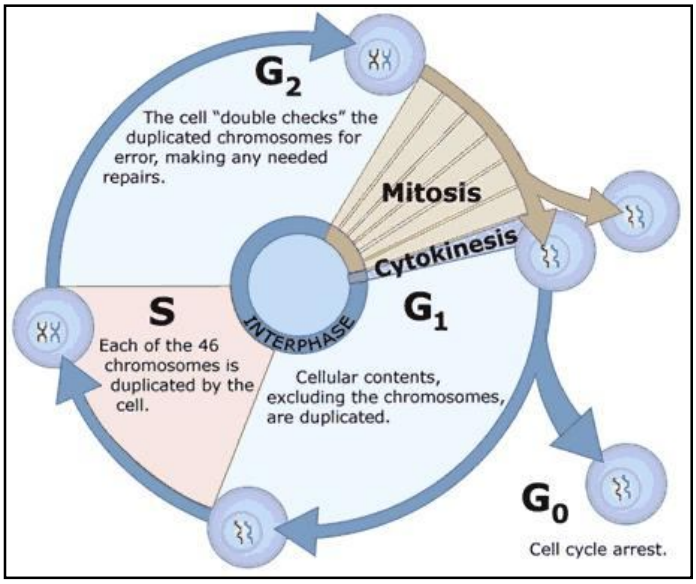

Figure: 3 Cell cycle

The nuclear DNA content of a cell can be quantitatively measured at high speed by flow cytometry. Initially, a fluorescent dye that binds stoichiometrically to the DNA is added to a suspension of permeabilized single cells or nuclei. The principle is that the stained material has incorporated an amount of dye proportional to the amount of DNA. The stained material is then measured in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell. Thereafter, such fluorescence data are considered a measurement of the cellular DNA

content. Samples should be analyzed at rates below 1000 cells per second in order to yield a good signal of discrimination between singlets or doublets. Since the data obtained is not a direct measure of cellular DNA content, reference cells with various amounts of DNA should be included in order to identify the position of the cells with the normal diploid amount of DNA. Some of the common reference cells often used for DNA measurements is human leukocytes or red blood cells from chicken and trout. Commonly DNA measurements are expressed as a DNA index of the ratio of sample DNA peak channel to reference DNA peak channel. A DNA index of 1.0 represents a normal diploid DNA content, while deviations in cellular DNA content values other than 1.0 indicate DNA aneuploidy.

The Analysis of the Cell Cycle In addition to determining the relative cellular DNA content, Flowcytometery also enables the identification of the cell distribution during the various phases of the cell cycle. Four distinct phases could be recognized in a proliferating cell population: the G1-, S- (DNA synthesis phase), G2- and M-phase (mitosis). However, G2- and M-phase, which both have an identical DNA content, could not be discriminated based on their differences in DNA content **(Figure-32).** Diverse software containing mathematical models that fit the DNA histogram of a singlet have been developed in order to calculate the percentages of cells occupying the different phases of the cell cycle.

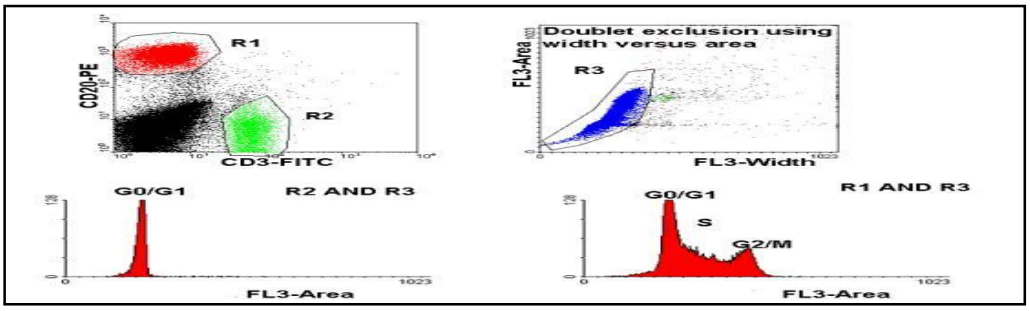

Figure:4 Cell cycle analysis

The advent of high speed, cell analysis systems for rapid measurement of physical and bio-chemical properties in single cells has provided new and useful techniques foe performing a wide variety of biological experiments. Such techniques have been used to determine the DNA content of a cell and the effect of chemotherapeutic agent on cell cycle traverse. Analysis of a population of cells' replication state can be achieved by fluorescence labeling of the nuclei of cells in suspension and then analyzing the fluorescence properties of each cell in the population. Quiescent and G1 cells will have one copy of DNA and will therefore have 1X fluorescence intensity. Cells in G2/M phase of the cell cycle will have two copies of DNA

and accordingly will have 2X intensity. Since the cells in S phase are synthesizing DNA they will have fluorescence values between the 1X and 2X populations.

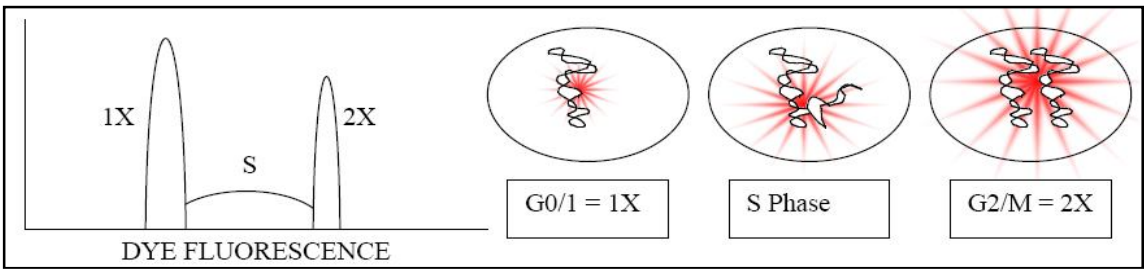

Figure: 5 Represents different phases in cell cycle analysis

The resulting histograms consist of three populations: two Gaussian curves (1X and 2X peaks) and the S-phase population. Adjacent populations overlap each other. Because of this, a modeling program is required to de-convolute the populations and assign percentage values to each population. Expert and subjective review of the modeling software's cell cycle phase percentage assignment is the final stage of cell cycle analysis prior to reporting the results.

Chemicals Required

- RNase (USb, lot No.-121691)
- Propidium iodide
- Ethanol (changshuyangyuan chemical, lot. No. 080220)
- Phosphate buffer saline (HIMEDIA, Lot No. TX027)
- Camptothecin (sigma, C-9911)
- SS-176 (IIIM, BOC Lab)
- SK-20 (IIIM, BOC Lab)

Apparatus Required

- 6 well plate (Nunc, Cat. No. 140675)
- 15 ml centrifuge tubes (Tarsons, Cat. No. 546020)
- Tissue culture flasks – 75 Cm^2 (T-75) (Nunc, Cat No.156499)
- 5ml Pipette (Sterilin Lot No.809407)
- Pipettes Dispenser (BRAND) (accu-jet$^{@}$pro)
- Tips for Micropipettes (2-20µl, 20-200µl, 200-1000µl) (Tarson)
- BD Falcon^{TM} tube (Cat. No.- 352003)

Instruments

- Flow cytometer (BD FACSAria II)
- Carbondioxide incubator (Heraeus, Germany)

- Centrifuge (Beckman,USA; G S-6R)
- Deep freezer (Scien Temp, USA)
- Hemocytometer (Sigma Chem. Co., USA, Cat No Z375357-13A)
- Vertical laminar flow - Clean air work station (Klenzaids Model No.1194)

Procedure

- The experiment was performed using HL-60 human cancer cell line. Cells were seeded in 6 well Plate the concentration of $2x10^6$cells/ml/well.
- Samples were added at desired concentration, sparing wells for negative and positive control.
- Plates were incubated in CO_2 incubator. After 18 hours of incubation Camptothecin (5 μM) was added to the well for positive control, and plates were incubated further for 6 hours in CO_2 incubator.
- After 6 hours incubation, material from each well was extracted using a micropippete and separately transferred into 15ml centrifuge tube.
- Tubes were centrifuged at 1200 rpm for 5 minute.
- The supernatant was discarded and pellet was resuspended in 1 ml filtered PBS and centrifuged at 1200 rpm for 5 minute.
- Supernatant was discarded and pellet was resuspended in 70% ethanol (ethanol can be added forcibly from a pipette or dropwise while vortexing).
- Cells were fixed for at least 24hour at 4ºC (Cells may be stored in 70% ethanol at -20ºC several weeks prior to PI staining and flow cytometric analysis).
- Cells were again centrifuged at 1200 rpm for 5 minutes.
- Cells were washed two times in filtered PBS by centrifuging at 1200 rpm for 5 minutes.
- Supernatant was discarded and tubes were placed in an inverted position over tissue paper till all the supernatant drained over the paper.
- Cells were washed with PBS, subjected to RNase digestion followed by staining of clean nuclear materials (nuclei) with propidium iodide using procedures and reagents as described in the instruction manual of the Cycle Test plus DNA reagent kit (Becton Dickinson, USA). The preparations were analyzed for DNA content using BD-FACSAria-II flowcytometer. Data were collected in list mode on 10,000 events for FL2-A vs. FL2-W. Apoptotic nuclei appear as a broad hypodiploid DNA peak at lower fluorescence intensity compared to nuclei in G_0/G1 phase.

3.7 MITOCHONDRIAL MEMBRANE POTENTIAL

The mitochondrial permeability transition is an important step in the induction of cellular apoptosis. During this process, the electrochemical gradient (referred to as $\Delta\Psi$) across the mitochondrial membrane collapses. The collapse is thought to occur through the formation of pores in the mitochondria by dimerized Bax or activated Bid, Bak, or Bad proteins. Activation of these pro-apoptotic proteins is accompanied by the release of cytochrome c into the cytoplasm, which promotes the activation of caspases, which are directly responsible for apoptosis.

Rhodamine 123, a fluorescence probe which selectively enters mitochondria with an intact membrane potential and is retained in the mitochondria, whose mitochondrial fluorescence intensity decreases quantitatively in response to dissipation of the mitochondrial membrane potential, was used to evaluate perturbations in mitochondrial membrane potential. The mitochondrial respiratory chain produces energy which is stored as an electrochemical gradient which consists of a transmembrane electrical potential, negative inside of about 180-200 mV, and a proton gradient of about 1 unit; this energy is then able to drive the synthesis of ATP, a crucial molecule for a consistent variety of intracellular processes. Several membrane permeable lipophilic cations, accumulated by living cells, organelles and liposomes exhibiting a negative interior membrane potential, have been used to study Mitochondrial Membrane Potential. Such probes include those which exhibit optical and fluorescence activity after accumulation into energized systems, such as 3, 3'-diehexiloxadicarbocyanine iodide [$DiOC_6$ (3)], nonylacridine orange (NAO), safranine O, Rhodamine-123 (Rh123) etc.

Chemicals Required

- Phosphate buffer saline (HIMEDIA, Lot No. TX027)
- Rhodamine 123 dye. (sigma)
- Camptothecin (sigma, C-9911)
- SS-176 (IIIM, BOC Lab)
- SK-20 (IIIM, BOC Lab)

Apparatus Required

- 6 well plate (Nunc, Cat. No. 140675)
- 15 ml centrifuge tubes (Tarsons, Cat. No. 546020)
- Tissue culture flasks – 75 Cm^2 (T-75) (Nunc, Cat No.156499)
- 5ml Pipette (Sterilin Lot No.809407)

- Pipettes Dispenser (BRAND) (accu-jet$^{@}$pro)
- Tips for Micropipettes (2-20µl, 20-200µl, 200-1000µl) (Tarson)
- BD Falcon^{TM} tube (Cat. No.- 352003)

Instruments

- Flowcytometer (BD FACSAria II)
- Carbondioxide incubator (Heraeus, Germany)
- Centrifuge (Beckman,USA; G S-6R)
- Hemocytometer (Sigma Chem. Co., USA, Cat No Z375357-13A)
- Vertical laminar flow - Clean air work station (Klenzaids Model No.1194)

Procedure

- The experiment was performed using HL-60 human cancer cell line. Cells were seeded in 6 well Plate the concentration of $1x10^{6}$cells/ml/well.
- Samples were added at desired concentration, sparing wells for negative and positive control.
- Plates were incubated in CO_2 incubator. After 18 hours of incubation Camptothecin (5 µM) was added to the well for positive control, and plates were incubated further for 5 hours in CO_2 incubator.
- After 5 hours incubation, add 10µl of Rhodamine 123 dye (conc. of 200nm) to each well in a sterile condition.
- After completion of 24hours incubation, material from each well was extracted using a micropipette and separately transferred into 15ml centrifuge tube.
- Tubes were centrifuged at 1200 rpm for 5 minute.
- The supernatant was discarded and pellet was resuspended in 1 ml filtered PBS and centrifuged at 1200 rpm for 5 minute for two times.
- Supernatant was discarded and pellet was resuspended in 500µl of PBS.
- Analyzed the intensity of Fluorescence from 10,000 events was in FL-1 channel on Flow cytometer.

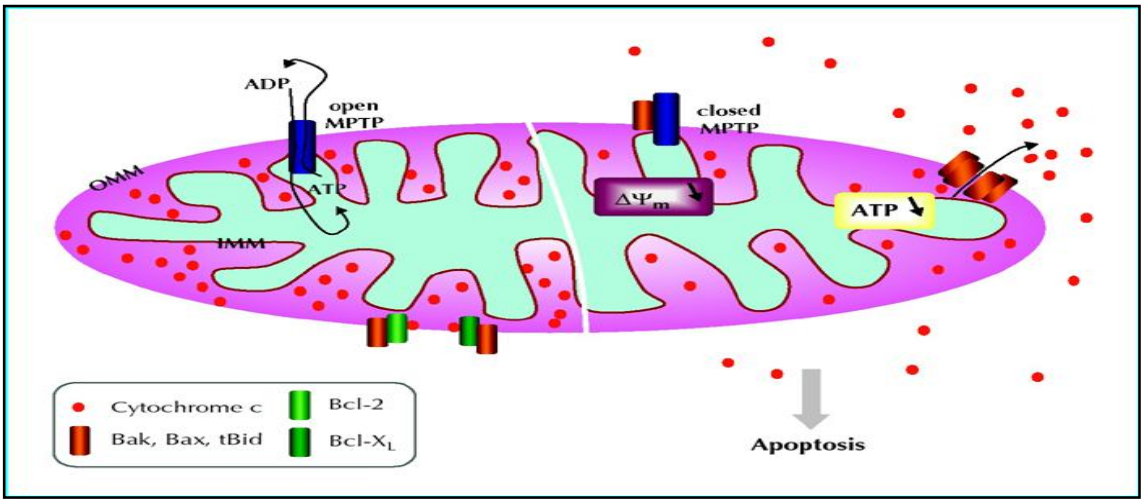

Figure: 6 Mitochondrial Membrane Potential (Mazure *et al.*, 2011).

3.8 WESTERN BLOT ANALYSIS

Preparation of Whole Cell Lysate for Immunoblotting

Cells ($3x10^6$) after treatment with test material were harvested and resuspended in 0.2ml of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 5 mM EDTA, 30 mM Na_2HPO_4, 50 mM NaF, 0.5 mM $NaVO_4$, 2 mM phenylmethylsulfonyl fluoride, and 10% protease cocktail inhibitor). Cells were incubated on ice for 30 min, vortexed and centrifuged at 12000xg for 15 min. Supernatants were collected and stored at -80^0C (**Han *et al.*, 2004)**. The protein contents were determined using Bradford reagent (Bio-Rad protein assay kit) and aliquots normalized to equal quantities before loading.

The protein lysates along with standard protein marker are subjected to discontinuous SDS-PAGE analysis. Proteins aliquots (50µg) are resolved on SDS-PAGE, run at 60V (PowerPac^{TM} HC High current power supply, BioRad), for 3h. The resolved proteins were electro transferred to polyvinylidene difluoride (PVDF) membranes (Bio-rad) in to Western bloting transfer frames in the following manner: Sponge-Blotting paper-gel-PVDF membrane-western blotting paper-sponge and transfer overnight at 4°C at 30V in transfer buffer. Non-specific bindings of the membrane are blocked by incubation with 5 % non-fat milk in Tris-buffered saline (10mM Tris-HCl, 150mM NaCl) containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The blots are probed with respective primary anti-human antibodies for 2 h (1:1000 dilutions) and washed three times with TBST. The blots are then incubated with horseradish peroxidase conjugated respective secondary antibodies for 1 h (1:1000 dilution), washed again three times with TBST. PVDF membrane was incubated in

to ECL Pus western blot detection reagent (ECL kit, Amersham Biosciences) for 5 min on a transparency sheet, in dark. PVDF membrane was placed in to the Hyper Cassette and superimposed with high performance chemiluminescence's film in the dark room, wait for the 2 min and develop the protein signal on to the high performance chemiluminescence's X-ray film by using developer and fix the signal by processing chemical fixer. Wash out the film with tap water gently and dry. The density of the bands was arbitrarily quantified using Quantity One software of Bio-RAD gel documentation system.

Protein Estimation

Bio-Rad protein assay kit solvent (5X) is diluted to 1X with HPLC grade water and filtered through whatman filter no 1. Add 190 µl of this reagent in to each well of 96 well plates and to this add 50µl of protein sample, mix and wait for 5 min. Read the absorbance at 595 nm on Elisa plate reader. A standard plot of BSA (1-100µg) is drawn using same procedure and slope of the straight line determined. Amount of sample protein is calculated through the standard calibration plot of BSA.

Preparation of Whole Cell Lysate for Immunoblotting

1. 2×10^6 cells were treated as desired.
2. Cell were collected and incubated in cold lysis buffer (50mM Tris pH 8.0, 150mM NaCl, 5mM EDTA, 1% v/v Nonidet P-40, 1mM PMSF and 1% (v/v) eukaryotic protease inhibitor cocktail) for 30 min on ice.
3. The lysates were centrifuged at 12 000g, 10 min, 4ºC and supernatant collected as whole cell lysate.

Materials

1. Cells – HL-60
2. PBS
3. RIPA buffer (Radio Immuno precipitation assay buffer) – 1400 µl :-

- Sodium fluoride –(50mM)
- PMSF (poly methyl sulfoxide) – 2mM
- Protease inhibitor cocktail - 20µl
- Sodium orthorandate – .5mM

4. Brad ford reagent
5. Loading dye (2X) :-
    - Bromophenol blue dye – 0.2% w/v
    - β- mercaptoethanol – 200mM
    - Glycerol – 20% v/v
    - SDS – 4% v/v
    - Tris.cl – 100mM
    - pH – 6.8
6. PVDF membrane (Poly vinylidene fluoride membrane)
    - Length – 8.75
    - Breadth – 6.5
7. Transfer buffer (1 X)
8. Blocking buffer (1X)
9. BSA (Bovine serum albumin)
10. Primary antibody
11. Secondary antibody

Gel electrophoresis

Separation of proteins was performed by denaturating SDS-polyacrylamide gel electrophoresis. This technique allows the electrophoretic separation of denaturated proteins according to their size. Sodium dodecyl sulfate (SDS), a highly negative charged detergent, solubilises proteins and leads to a constant net charge per mass unit. Hence, SDS-polypeptide complexes migrate toward the anode through the polyacrylamide gel according to their molecular weight. In addition, the differences in molecular shape are compensated by the loss of the tertiary and secondary structures because of the disruption of the hydrogen bonds and unfolding of the molecules. By adding of a reducing agent like dithiothreitol (DTT) disulfide

bonds are cleaved and proteins are totally unfolded. The molecular weight of the investigated proteins is estimated by applying molecular weight standards (ChemichromeTM western control, Sigma). Discontinuous gel electrophoresis is commonly used.

The most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS). SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure and thus allows separation of proteins by their molecular weight.

The gels generally consist of acrylamide, bisacrylamide, SDS, and a Tris-Cl buffer with adjusted pH. The solution is degassed under a vacuum to prevent air bubbles during polymerization. [1]Ammonium persulfate and TEMED are added when the gel is ready to be polymerized. The separating or resolving gel is usually more basic and has a higher polyacrylamide content than the loading gel. Gels are polymerized in a gel caster. First the separating gel is poured and allowed to polymerize. Next a thin layer of isopropanol is added, causing the top of the separating gel to form a smooth surface. Next, the loading gel is poured and a comb is placed to create the wells. After the loading gel is polymerized the comb can be removed and the gel is ready for electrophoresis.

Samples are loaded into wells in the gel. One lane is usually reserved for a marker or ladder, a commercially available mixture of proteins having defined molecular weights, typically stained so as to form visible, coloured bands. When voltage is applied along the gel, proteins migrate into it at different speeds. These different rates of advancement (different electrophoretic mobilities) separate into bands within each lane.

The electrophoresis apparatus is set up with cathode buffer (SDS, Tris, Tricine, and distilled deionized water) covering the gel in the negative electrode chamber, and anode buffer (Tris-Cl, distilled deionized water and is adjusted to a higher pH than the cathode buffer) in the lower positive electrode chamber. Next, the denatured sample proteins are added to the wells one end of the gel with a syringe or pipette. Finally, the apparatus is hooked up to a power source under appropriate running conditions to separate the protein bands.

Procedure

1. Proteins from different samples were separated by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli.
2. The gel plates, combs, spacers were washed and then cleaned with 70% ethanol to remove any grease.
3. The gel cassette was formed by fitting spacers at each edge, and then the unit was clamped together.
4. The separating gel was loaded, so that top of the gel was approximately 4.5cm from the top of notched end of the glass plate. Immediately, the gel was overlaid with isopropanol, taking care to exclude any air bubbles.
5. The gel was allowed to polymerize for 30-45 min.
6. The layer of isopropanol on the top of separating gel was removed after the gel polymerized.
7. The stacking gel was prepared an.d loaded on the top of separating gel.
8. Immediately, a comb was inserted into the stacking gel to form the wells and water was overlaid over the stacking gel to prevent evaporation. This was left to set for 30 min.
9. The comb was removed gently and the wells were washed with DDW to remove the catalyst or any unpolymerized components.
10. The plate was placed in the electrophoresis apparatus and the leads were connected
11. Samples were mixed with sample buffer in 4:1 ratio and then placed in heating block at 70 ^{o}C for 15 minutes. Samples containing 50µg of protein were loaded per well.
12. The chemichrome western control was loaded in the first well so as to identify the band of interest during western blotting.
13. The top and bottom well units were filled completely with reservoir buffer and the power supply was connected.
14. Initially gel was run at 50 V for 20min. After the protein crossed the stacking gel, the current was increased to 100 V and it was run for 130 min.

RESOLVING GEL FOR SDS-PAGE (10ml)

COMPONENTS	**6%**	**8%**	**10%**	**12%**
H_20	5.3	4.6	4.0	3.3
30% acrylamide – bisacrylamide	2.0	2.7	3.3	4.0
1.5M Tris (PH 8.8)	2.5	2.5	2.5	2.5
10% SDS	0.1	0.1	0.1	0.1
10% APS	0.1	0.1	0.1	0.1
TEMED	0.008	0.006	0.004	0.04

STACKING GEL FOR SDS-PAGE (5%)

COMPONENTS	**6 ml**	**8 ml**	**10 ml**	**16 ml**
H_20	4.1	5.5	6.8	11
30% acrylamide – bisacrylamide	1.0	1.3	1.7	2.6
1.5M Tris (PH 6.8)	0.75	1.0	1.25	2.0
10% SDS	0.06	0.8	0.1	0.16
10% APS	0.06	0.8	0.1	0.16
TEMED	0.006	0.08	0.01	0.016

Transfer of bands from gel to PVDF membrane

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF). Take PVDF membrane (length-8.75cm,breadth-6.5cm), dissolve this in methanol for 2 min to remove hydrophobicity, then dissolve in transfer buffer for 10-20 min. Now take out the gel and cut the stacking gel. The membrane is placed on top of the resolving gel, and a stack of filter papers placed on top of that. The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it. This can be done for 2 hrs at 70V.

The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with Coomassie Brilliant Blue or Ponceau S dyes. Ponceau S is the more common of the two, due to Ponceau S's higher sensitivity and its water solubility makes it easier to subsequently destain and probe the membrane.

Blocking

Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically 3-5% Bovine serum albumin (BSA) or non-fat dry milk (both are inexpensive) in Tris-Buffered Saline (TBS), with a minute percentage of detergent such as Tween 20 or Triton X-100. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein.

DETECTION

Primary antibody: Primary antibodies are generated when a host species or immune cell culture is exposed to the protein of interest. After blocking, a dilute solution of primary antibody (generally between 0.5 and 5 µg/mL) is incubated with the membrane under gentle agitation. Typically, the solution is composed of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA. The antibody solution and the membrane can be sealed and incubated together for anywhere from 3 hrs to overnight, then wash it 3 times with 1 X TBST. It can also be incubated at different temperatures, with warmer temperatures being associated with more binding, both specific and non-specific.

Secondary antibody: After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. Antibodies come from animal sources (or animal sourced hybridoma cultures); an anti-mouse secondary will bind to almost any mouse-sourced primary antibody, which allows some cost savings by allowing an entire lab to share a single source of mass-produced antibody, and provides far more consistent results. This is known as a secondary antibody, and due to its targeting properties, tends to be referred to as "anti-mouse," "anti-goat," etc. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal. Secondary antibody is added for 1 -2 hr and then wash it 3 times with 1X TBST for 15 min.

The blots were probed with respective mouse primary antibodies for 2 h and washed three times (5 minutes each wash) with TBST. The blots were then incubated with horseradish peroxidase conjugated or rabbit anti mouse secondary antibodies for 1 h, washed again three times (15 minutes each wash) with BST and signals detected using ECL plus chemiluminescence's kit on X-ray film. The protein contents were determined using Bradford reagent (Bio-Rad protein assay kit) and aliquots normalized to equal quantities before loading.

3.9 HOECHST 33258 NUCLEAR STAINING

In this method, the staining solution (10 µg/ml, Hoechst 33258, 0.01 M citric acid and 0.45 M disodium phosphate containing 0.05% Tween-20) is used and stained for 30 min in the dark at room temperature.

Procedure

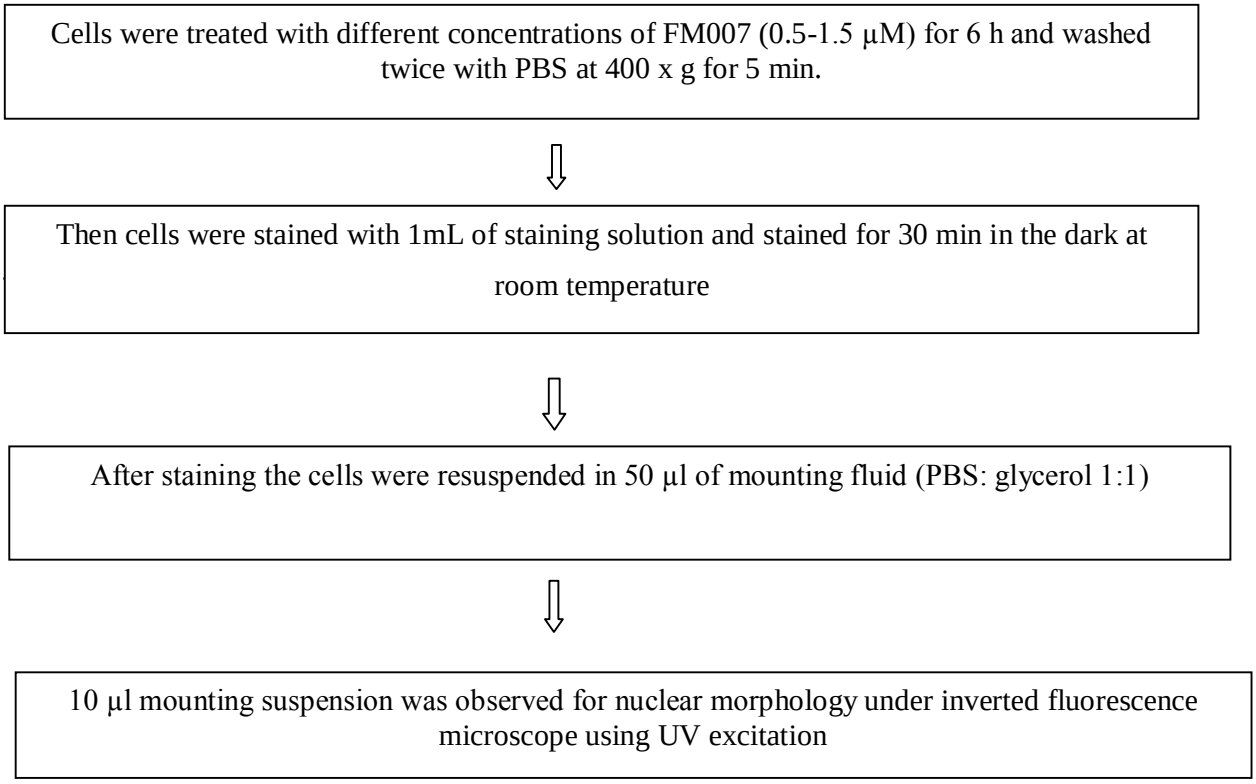

Figure 22: Protocol of Hoechst Staining (Bhushan S *et al*., 2006).

Chapter-4

RESULTS

4.1 CELL PROLIFERATION ASSAY

Inhibition of proliferation was demonstrated through MTT assay. The IC_{50} values were calculated for different human cancer cell lines for 48h and found THE IC_{50} value was very low in HL-60 cell line 0.9 µM. therefore we decided our further studies in HL-60 cell line.

Compounds	% Growth Inhibition at 100µM in Different Human Cancer Cell Line (IC50, µM)					
	HL-60	PC-3	Mia-Paca-2	MCF-7	HUVEC	T47D
NPC001	1	58	1.8	31	4.4	3.7
NPC002	3	30	0.8	8	8	8
NPC005	8	31	1.6	49	39	5.6
NPC007	0.9	41	0.6	4	8	7
NPC008	4	>100	4	4.7	4.3	NT
NPC009	0.4	10	3	2.4	6	17

4.2 PHASE CONTRAST MICROSCOPY

External morphological changes were observed through phase contrast microscopy and NPC-007 was found to induce cellular damage in a dose-dependent manner.

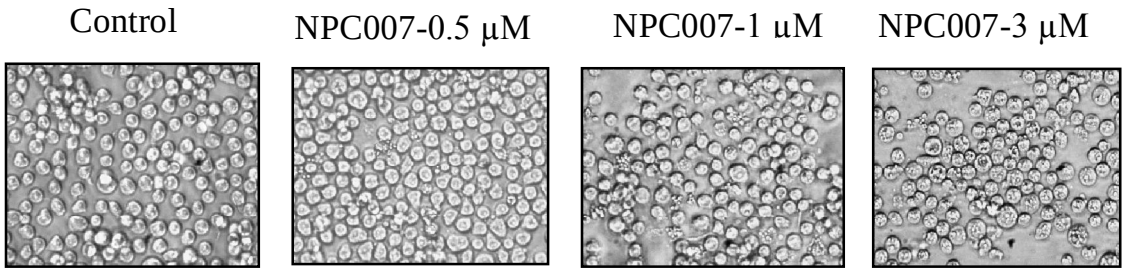

Figure:1 HL-60 cells were incubated with 0.5 µM,1 µM and 3 µM of NPC007 for 24h. Phase contrast microscopy was done to observe the morphological changes in cells after treatment with NPC007. NPC007 showed the changes in morphology in a dose dependent manner. Phase contrast microscopy treated and untreated cells were photographed using compound microscope, Olympus IX70.

4.3 HOECHST 33258 STAINING OF CELLS

NPC007 was also found to damage DNA in dose-dependent manner as indicated by increased number of apoptotic bodies' formation in nuclei observed by Hoechst staining. DNA damage is considered as hallmark of apoptosis and significant DNA damage was observed through Hoechst staining.

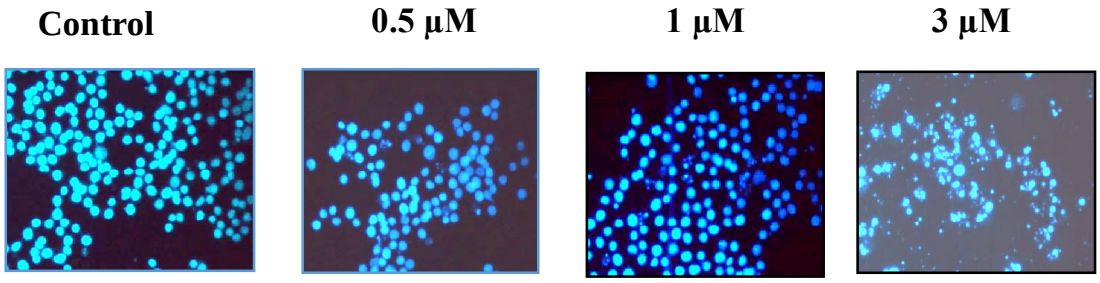

Fig.2 Hoechst staning of NPC007 treated Human leukemia HL-60 cells. Cells were incubated with indicated concentrations of NPC007 for 24 h time period. Control cells have round and healthy nuclei while treated cells showed condensed nuclei wrapped in apoptotic bodies as indicated by white arrows in the figure, which increase as we proceed from lower to higher concentration of compounds. For Phase contrast microscopy treated and untreated cells were photographed using compound microscope, Olympus IX70.

4.4 CELL CYCLE ANALYSIS

DNA fragmentation constitutes one biochemical hallmark of apoptosis. Thus, measurement of DNA content makes it possible to identify apoptotic cells, to recognize the cell cycle phase specificity and to quantitate apoptosis. For flow cytometry analysis of the relative nuclear DNA content the fluorescent dye propidium iodide (PI), which becomes highly fluorescent after binding to DNA, is most commonly used. After permeabilization PI binds to DNA in cells at all stages of the cell cycle, and the intensity with which a cell nucleus emits fluorescent light is directly proportional to its DNA content. The results of the measurement are illustrated in a histogram, where the number of cells (counts) is plotted against the relative fluorescence intensity of PI (FL-2; λem: 585 nm; red fluorescence).

Measurement of DNA content makes it possible to identify apoptotic cells and cell cycle phase specificity. NPC007 was earlier reported as a potent CDK4 inhibitor, arresting the cell cycle in G1 phase , but when HL-60 cells were treated with NPC007 for 24 h, a concentration-dependent rise in hypo-diploid sub-G0 DNA fraction (apoptotic, <2nDNA) and S-phase arrest was observed. The sub-G0 fraction was 1% in control cells, which increased to 98% after 3 µM of NPC007 treatment, whereas S-phase fraction was 56% in untreated and NPC007 treated HL-60 cells, respectively. Thus, NPC007 seems to be cytotoxic agent as it

inhibits S-phase and sub-G0 arrest in the cell cycle analysis. Cytotoxic agents, at both high and low drug concentrations delay cell progression and induce lethality in S-phase, whereas, acytotstatic agents delay cell progression in G1 phase, without lethality at intermediate drug concentrations **(Rixe et al,2007).**

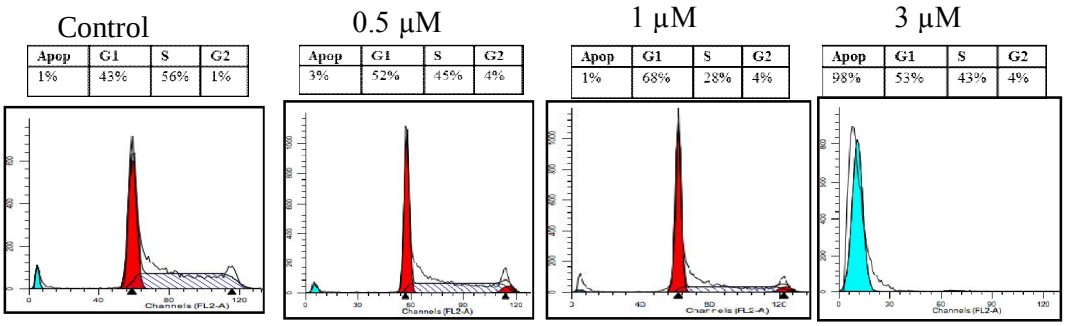

Fig. 3 Compound NPC007 increased hypo diploid Sub-G0 cell population in human leukaemia HL-60 cells. Cells (0.44×10^6/ml/24 well plate) were treated with NPC007 for 24 h at indicated concentrations. Cells were stained with PI for 30 min to determine DNA fluorescence by flow cytometery as described in material and methods. Sub-G0 DNA population here is an indicative of DNA damage was analysed from the hypo diploid fraction (<2*n* DNA) of DNA cell cycle analysis using Mod Fit software. Data are representative one of three similar experiments.

4.5 MITOCHONDRIA MEMBRANE POTENTIAL (MMP ANALYSIS)

Mitochondrial integrity is required for cell to be healthy and mitochondrial membrane potential loss is considered as one of the most important sign of the apoptosis. We checked the MMP loss by NPC007 and found that NPC007 significantly induced MMP loss. The loss increased with increasing doses of NPC007.

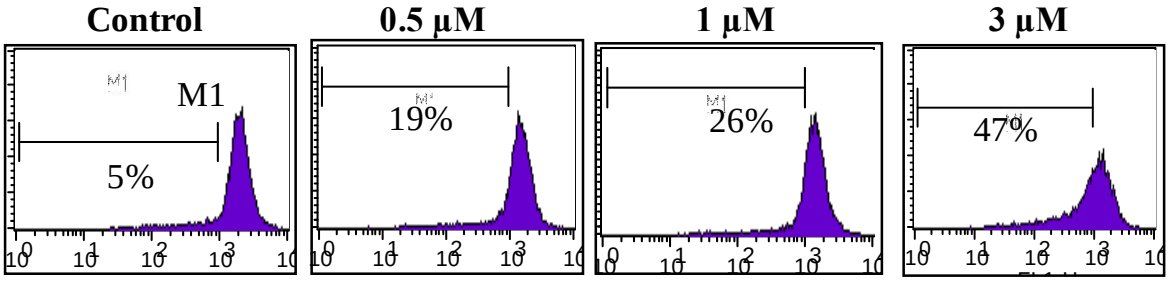

Fig.4 NPC007 induced loss of mitochondrial membrane potential ($\Delta\psi_m$) in HL-60 cells. Cells at 30-40% confluent stage were incubated with indicated concentration of Sample NPC007 for 24 hours. Thereafter Rhoamine-123, added 40min before analysed in the FACS.

NPC-007 induces loss of mitochondrial membrane potential- Aerobic glycolysis is the most common feature of cancer cells and elimination of mitochondrial DNA reduces the tumourogenicity of cancer cells (Douglas, 2012). Mitochondrial potential loss triggered by NPC-007 in HL-60 cells was analyzed by studying the uptake of Rhodamine-123 dye (Rh-123). Damaged cells have low uptake of the Rh-123 dye and hence low fluorescence. We have excluded the nonviable cells by applying the gate on viable cells during flowcytometer

data analysis. NPC-007 HL-60 cells showed 19, 26 and 47% mitochondrial potential loss at .5,1 and 3µM concentration respectively(Fig. 4).

Mitochondrial dysfunction triggered by NPC-007 is associated with translocation of mitochondrial apoptotic proteins- Mitochondria play a key role in activation of apoptotic or nonapoptotic cell death. Bcl-2 member family proteins translocate from mitochondria to cytosol during apoptotic induction. NPC-007 significantly inhibits the expression of anti-apoptotic protein Bcl-2 (Fig. 5A). While pro-apoptotic protein Bax expression was increased.

NPC-007 induced extrinsic and intrinsic apoptotic signaling *in* HL-60 cells- We further explored the mechanistic insights of NPC-007 in HL-60 cells. It inhibited the expression of pro-caspase 3,8 and 9 in HL-60 cells in a concentration-dependent manner (Fig. 5A). Both these extrinsic and intrinsic apoptotic events merged on caspase-3 activation at 3µM concentration, which resulted in the PARP cleavage and activation. (Fig. 5A).

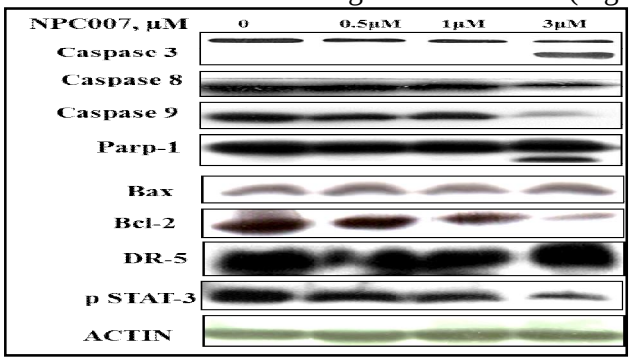

Figure 5A: After western blotting, PVDF membrane was developed and after that X-ray film was developed in dark room. Presence of caspase3, caspase8, caspase9 and prap-1indicate that this sample can cause MMP formation and lead to apoptosomes formation which will lead to apoptosis.

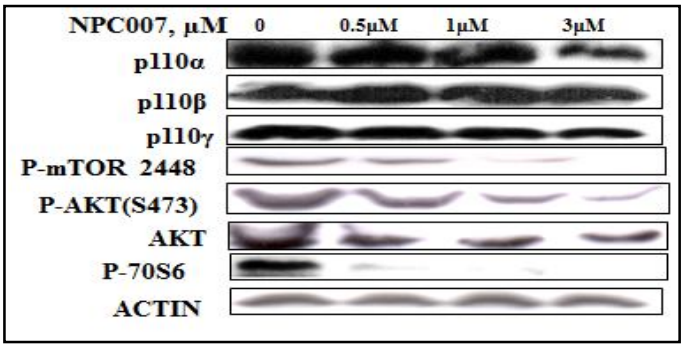

Figure 5B :

Inhibition of PI3K/Akt/mTOR pathway by NPC-007- PI3 K/Akt/mTOR pathway is responsible for the proliferation of the majority of cancers. The catalytic subunit of type I PI3K, p110a is mutated in the majority of cancers. NPC-007 inhibited the expression of p110-a,β and γ in a concentration dependent manner in HL-60 cells. (Fig.5B). It also inhibited the expression of Akt and its activated form (p-Akt, Serine 473) in a dose-dependent manner (Fig. 5B). Akt is a serine threonine kinase, which affects downstream signalling, that is involved in cell proliferation and growth. Similarly mTOR expression was also inhibited with increase in concentration of NPC-007. The mTOR is nutritional sensor of the cell, downstream to Akt **(Wullschleger et al., 2006).** It is mutated in various types of cancers and targeting mTOR is also a hot area in target-based anticancer studies.

Chapter-5

DISCUSSION

Cancer is preceded only by cardiovascular and infectious diseases as the leading causes of death worldwide. Its cure is still a challenge for the medical world. Leukaemia represented the sixth leading cause of death in the United States in 2013 and can affect people at any age **(Siegel et al., 2013).** Natural products have always been preferred as a source of novel human therapeutics for decades, and as a result more than 75% of the drugs in the market are from natural products. Now the aromatherapy or the use of essential oils in the cure of various diseases has become an attractive choice in recent years. Essential oils are used as antiviral, anti- bacterial or antifungal agents but reports regarding their antitumor activity have also been published in recent years **(Kumar et al.,2008; Cha et al., 2009; Torres et al., 2011; Bakkali et al., 2008)**. Due to their low toxicity, aromatic nature and abundant availability it may be an attractive candidate for the treatment of different cancers. In this work, we tried to explore the analysis of NPC007, a chalcone derivative in induction of apoptosis and inhibition of PI3K/Akt/mTOR pathway in human leukaemia HL-60 cells.

We are reporting for the first time the mechanism of action of apoptosis via influencing the PI3K/Akt/mTOR signalling cascade in human leukaemia HL-60 cells by NPC007. Our study demonstrated that NPC007 inhibited the cell viability of various cancer cell lines like HL-60,PC-3, MCF-7, MIA-PaCa-2 and T47D. Therefore, we were interested in finding out the reason behind this. So we explored the pro-apoptotic effect of NPC007 in HL-60 cells. We explored the apoptosis by means of various biological endpoints like appearance of apoptotic bodies and increase in the subG0 DNA fraction. Then we studied possible early events, which may be associated with apoptosis and evaluated the extrinsic and intrinsic pathways of apoptosis and other major supportive pathways that may be linked like PI3K/Akt/mTOR signalling cascade.

Mitochondria and Bcl-2 family of proteins play a pivotal role in the induction of apoptosis **(Douglas, 2012).** Bcl-2 associated proteins have both pro-apoptotic and anti-apoptotic effects in cancer cells. These proteins regulate mitochondrial outer membrane potential and control the release of many apoptotic factors originating in the mitochondria. NPC007 decreased the expression of mitochondrial associated anti-apoptotic proteins Bcl-2 in a concentration-dependent manner (Fig. 6). The down regulation of these two antiapoptotic proteins changes the symmetry of mitochondria and activates the mitochondrial permeability

transition pores (PTP) and induces the loss of mitochondrial membrane potential (Wmt). Loss of mitochondrial membrane potential is an early apoptotic event and damaged mitochondria relay many signals to downstream elements that initiate intrinsic apoptotic death-signalling in cells. NPC007 treated HL-60 cells show mitochondrial membrane potential loss. (Fig.4). The loss of mitochondrial membrane potential further triggers the apoptotic machinery via release of pro-apoptotic factors such as cytochrome c and Bax. During apoptosis, Bax is translocated to the mitochondria and creates pores in the mitochondrial outer membrane which results in an increase in mitochondrial permeability **(Nechushtan et al., 2001).**

NPC-007 induce loss of mitochondrial potential, therefore we studied which activate caspase-9, caspase-3 and PARP-1 cleavage **(Jiang and wang, 2004**). The role of caspase in apoptosis is well established and active caspase-8 or caspase-9 activatevarious effector caspases including caspase-3, -6, and -7, which results in the enhanced expression of protease activity in the cell and cleavage of various5 proteins like PARP-1 **(Strasser et al., 2000; Rastogi et al., 2009).** As NPC-007 activates both caspase-3 and PARP-1, therefore, we can conclude that they induce apoptosis via extrinsic activation pathways in HL-60 cells.

Next, we explored the effect of MC and thymol on other major supportive pathways linked to apoptosis like PI3K/Akt/mTOR signaling cascade. We are reporting for the first time that NPC-007 inhibits the PI3K/Akt/mTOR signaling in human leukaemia7 HL-60 cells. The discovery of novel PI3K/AKT/mTOR inhibitors is highly intriguing because of the significant altered expression of these proteins in a variety of tumors, which make them an attractive target for anti-cancer therapy. In recent years, extensive efforts have been made to discover inhibitors of the PI3K/AKT/mTOR pathway for the treatment of cancers and several of these inhibitors such as viz. NVP-BEZ-235, GSK-690693, PKI-587, XL-765 and PI-103 are being evaluated in clinical trials. The PI3K/AKT/mTOR pathway regulates several cellular functions that are also critical for tumor genesis such as cell proliferation, cell metabolism, angiogenesis, cell cycle progression, apoptosis and autophagy **(Hennessy et al., 2005; Courtney et al., 2010)**. PI3 Kinases transmit extracellular signals into cells by generating phospholipids, which cause the phosphorylation and activation of Akt, this relays signals to mTOR which acts as a sensor for nutrient availability and is involved in cell growth **(Wullschleger et al., 2006).** mTOR is a serine/threonine kinase that stands in a central position on the cross road for various cell signal pathways (Ras, PI3K/Akt, VEGF, HIF) **(Hay and Sonenberg, 2004).**

Over activation of mTOR downstream p70S6K and eIF4E is frequently associated with activation of hypoxia inducing factor (HIF) which regulates tumor genesis,

angiogenesis and tumor growth through VEGF **(Semenza, 2003).** It is necessary for tumor angiogenesis and macroscopic solid tumor growth. NPC-007 inhibited the expression of p110a, Akt, p-Akt (S-473), mTOR, p-mTOR, p70S6K. As noted earlier in other cell death events the degree of inhibition of the PI3K/Akt/mTOR pathway by NPC007 in HL-60 cells.

CONCLUSION

The current study highlights the importance of Natural product Chalcone derivative in the discovery and development of novel anticancer agent. The derivative of Chalcone NPC-007 we are reporting here for the first time that it inhibit the PI3K/Akt/mTOR pathway and induce apoptosis via extrinsic pathways in human leukemia HL-60 cells. Hence the potential for NPC-007 may be highly encouraging and further studies are certainly warranted by the scientific and medical communities for its utilization as a possible future chemotherapeutic agent.

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