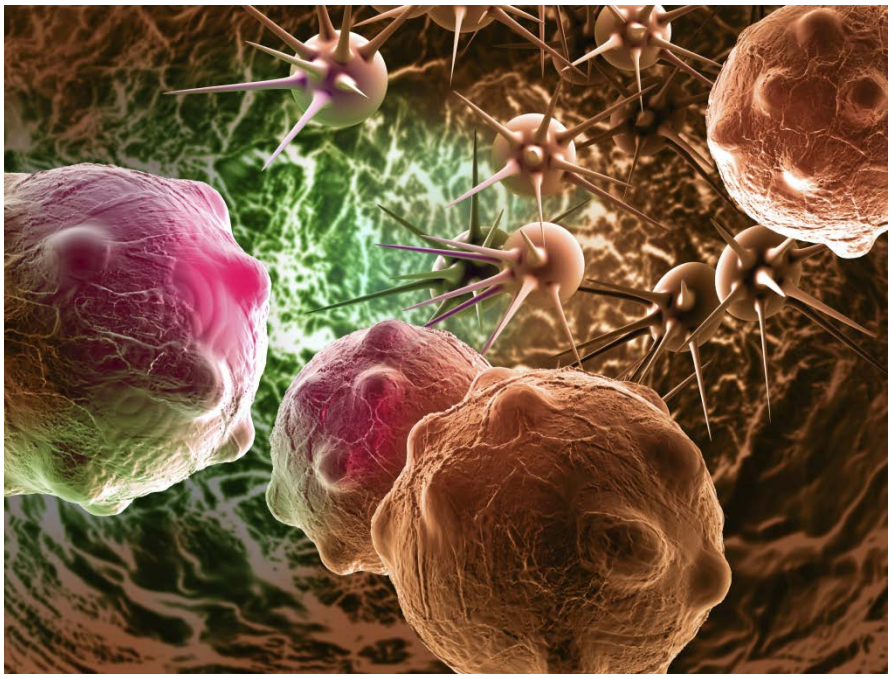
Chapter 1



Introduction

CHAPTER 1: INTRODUCTION

1.1 Cancer

The severity and impact of cancer on human health does not need any introduction in the present age. It keeps a prominent place in life threatening diseases of human beings and is the second leading cause of death worldwide. Its prevalence is worth notable to all regions around the globe. Cancer is regarded as an anthology of multiple diseases which are characterized by undifferentiated and continuous growth of certain cells of the body which further start invading adjacent tissues (Bhatia *et al.,* 2019; Weir *et al.,* 2016). The progression of this ailment can begin at any place of the body which is comprised of uncountable cells. Normal human cells grow and divide to produce newer cells according to the body's requirements. After certain age, old/damaged cells die and newly produced cells perform at their place (Chow, 2010; Charmsaz *et al.,* 2019). But development of cancer disrupts this natural process and instead of dying, the old or damaged cells remain survived and there is huge formation of unwanted cells (Gonzalez *et al.,* 2018). These unwanted cells further multiply without any interruption and form tumors (Zaitceva *et al.,* 2021). Maximum cancers lead to formation of solid masses and called as solid tumors whereas a few blood cancers like leukaemia do not form solid masses (Hao *et al.,* 2019).

A wide variety of tumors are malignant in nature as they have tendency to spread and invade the surrounding tissues (Martin *et al.,* 2013). Even these tumor cells can arrive up to distant parts inside the body through blood or lymph leading to development of new tumors away from the original tumors (Sainz *et al.,* 2012). Certain tumors are benign and do not invade the adjacent tissues. Instead, these are large and do not grow back after their removal, whereas malignant tumors may regenerate (Henske, 2003). Depending upon the pathogenesis and organ involved, cancers are of more than hundred varieties (Fares *et al.,* 2020). The major cause of cancer development is associated with genetic abnormalities of somatic cells leading to modifications in DNA (Stratton *et al.,* 2009; Takeshima *et al.,* 2019). These abnormalities lead to progression of cancer due to involvement of specific tumor susceptible genes which are namely gatekeepers, caretakers and landscapers (Kinzler *et al.,* 1997; Bissell *et al.,* 2001; Vogelstein *et al.,* 2002; Rajagopalan *et al.,* 2003). Gatekeepers include pro-oncogenes and suppressor genes which regulate normal cell growth and any alteration in this leads to cancer. Caretakers provide genetic stability and mutation in them

causes cancer initiation whereas alterations in landscapers are responsible for conversion of normal cells into tumor cells (Seiber *et al.,* 2003).

According to WHO fact sheets, approximately 9.6 million deaths have been reported due to cancer around the globe. It is evident that 70% of these deaths were related to developing and low income countries (Pilleron *et al.,* 2018; Lievens *et al.;* 2017). The prominent causes identified for occurrence of cancer were high body mass index, excessive tobacco consumption, alcohol intake, least physical activity and least intake of fruit and vegetables. Approximately 22% deaths were solely reported due to tobacco intake (WHO Fact Sheet July, 2021, www.who.int). Associated infections responsible for cancer were hepatitis and human papilloma virus. It was also notable that lung, prostate, colorectal and liver cancer were primary in men whereas women were found suffering greatly from breast, colorectal, lung and thyroid cancers (Sung *et al.,* 2021). According to 2017 statistics, only 30% of low income countries were having treatment services whereas in high-income countries these services were greater than 90%. In a nut shell, the burden of this ailment is continuous and growing rapidly all over the world, imposing huge physical, mental and economic stress on humans, societies and health systems. A huge population of low income countries is still unable to get timely diagnosis and cure to cancer even sometimes they are not able to afford the financial burden of treatment leading to reduced survival rate. The major causes of cancer have been presented in Figure 1.1 (Seigel *et al.,* 2021).

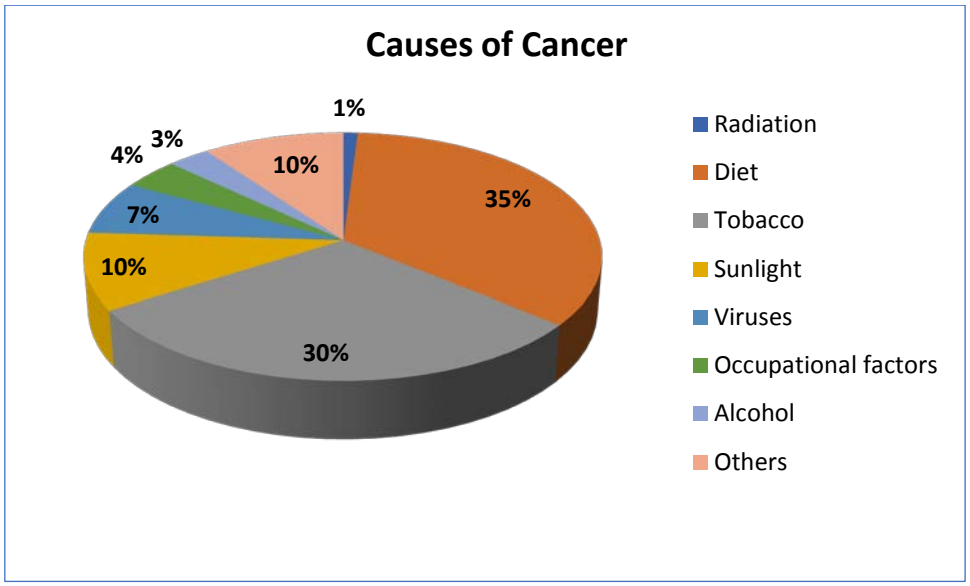


Figure 1.1: Prominent causes of cancer

1.1.1 Types of cancers

Cancers can be classified in several ways such as on the basis of site of origin or tissues involved. Common cancers on the basis of origin include bladder cancer, lung cancer, breast cancer, colon cancer, rectal cancer, kidney cancer, leukaemia, lung cancer, liver cancer, endometrial cancer, melanoma, Non-Hodgkin's lymphoma, pancreatic cancer, prostate cancer and thyroid cancer (Cronin *et al.*, 2018; Mathur *et al.*, 2020; Harris *et al.*, 2010). Classification based on tissues involved has been depicted in Table 1.1 (Imran *et al.*, 2017; Malone *et al.*, 2020; Waldman *et al.*, 2020).

Table 1.1: Types of cancer on the basis of tissues involved

S. No.	Type of Cancer	Description
1.	Carcinoma	Involves cells that form skin and tissue linings of the organs. These present abnormal growth of cells and are able to spread into adjacent tissues.
2.	Sarcoma	Arises from cells mf mesenchymal connective tissue including bone, cartilage, fat, vascular, or hematopoietic tissues.
3.	Lymphoma	Associated with cancer arising from immune-cells lymphocytes and may involve lymph nodes, spleen, thymus, bone marrow, and other parts of the body.
4.	Leukaemia	Occurs in blood forming tissues together with bone marrow. It presents elevated number of white blood cells which further replace healthy RBCs and platelets.
5.	Myeloma	Associated with antibody producing plasma cells. The infected cells further get accumulated in bone marrow and replace the healthy cells. This is rare type of cancer.
6.	Germ cell tumors	Associated with germ cells of gonads (testes and ovary). These tumors are present as outgrowth on the outer side of gonads and may be cancerous or non-cancerous in nature.

1.1.2 Pathogenesis of cancer

The pathophysiology of cancer involves a common pathway for development of cancer irrespective of type of cancer in an organism. The universal principle of pathogenesis of cancer has been described as genetic material damage through various events like mutation, disturbed gene expression, tumor promoter gene activation and defects in suppressor genes (Esteller, 2002; Bayllin *et al.*, 2016; Lowe *et al.*, 2000). All these events are involved in development and progression of malignant tumors. It is worth notable that cancer development involves various complex events which include mutation and identification of cells with rising ability for proliferation, survival, invasion, and metastasis (Collavin *et al.*, 2019; Jiang *et al.*, 2015; Guan *et al.*, 2015). The general etiology and pathogenesis of cancer has been outlined in Figure 1.2.

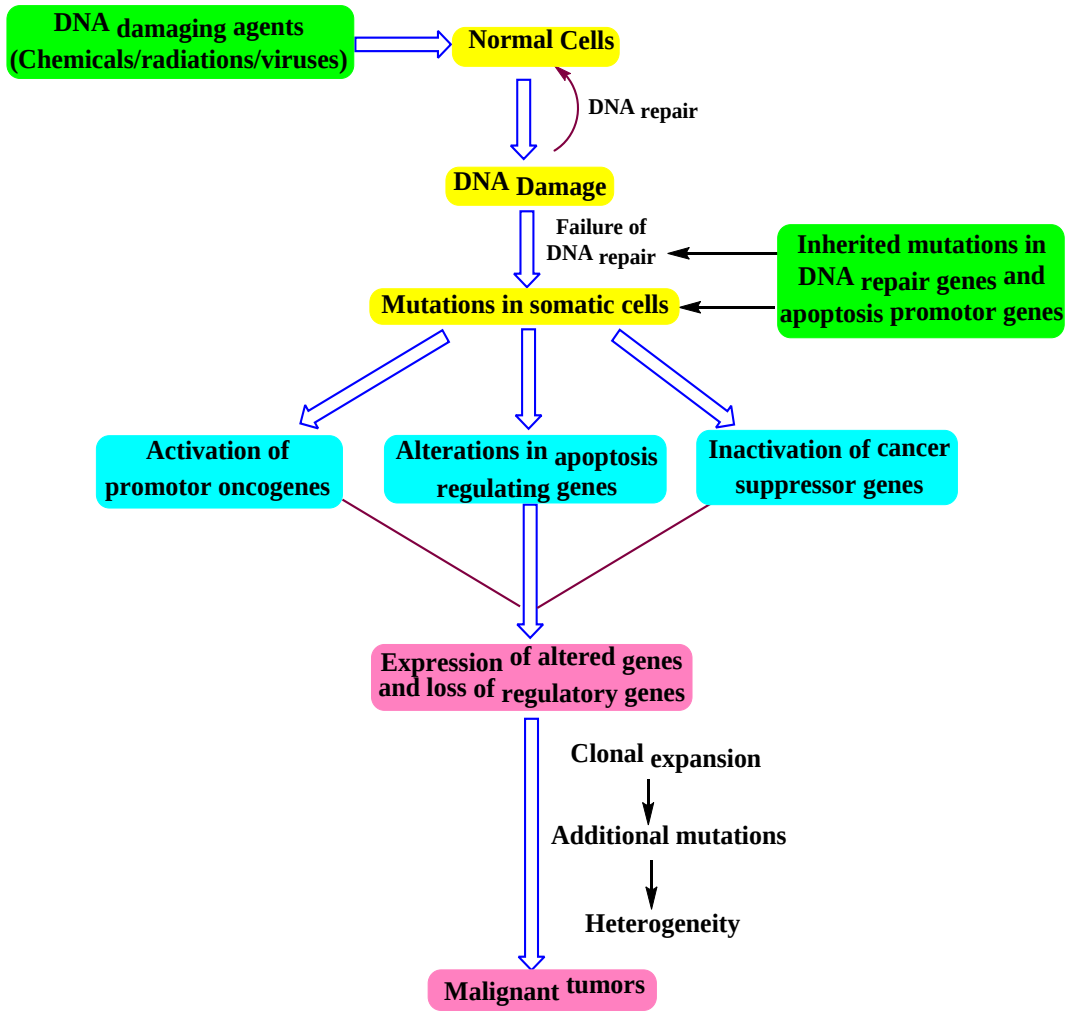


Figure 1.2: Etiology and pathogenesis of cancer

The progression of cancer can be summarized into following steps:

a. Mutation and initiation of tumor

Alteration in the genetic materials results into mutation in single cell which leads to anomalous proliferation of this cell producing tumor cells (Bajaj *et al.,* 2020).

b. Progression of tumor

With further mutations, tumor progression continues and increases the population of tumor cells. These tumor cells grow and divide more rapidly as compared to normal cells. The offspring of cell deportment such supplementary mutation becomes dominant within the tumor inhabitants (Pelosi *et al.,* 2019).

c. Malignancy and clonal selection

Tumor cell proliferation further forms tumor cell clones with extraordinary properties of rapid growth, survival, invasion and metastasis. This process is called as clonal selection which continues right through the tumor growth leading to more rapid tumor growth and development of malignancy (Greaves *et al.,* 2012).

d. Metastasis

Metastasis involves detachment of cancerous cells from the primary tumor and their migration to blood and lymphatic systems and ultimately arrival at various sites of the body (Fares *et al.,* 2020). These cells further multiply at new places and form newer tumors that reflect the tissue of origin. This metastatic property of tumors is the primary cause of their lethality (Hapach *et al.,* 2019].

1.1.3 Treatment strategies against cancer

Advancements in healthcare systems have led to development of several treatment strategies against cancer. The type of treatment is dependent on type/stage of cancer, general health of the patient and preferences. Various available treatment strategies against cancer have been presented in Figure 1.3 (Arruebo *et al.,* 2011; Topham *et al.,* 2016; Pucci *et al.,* 2019).

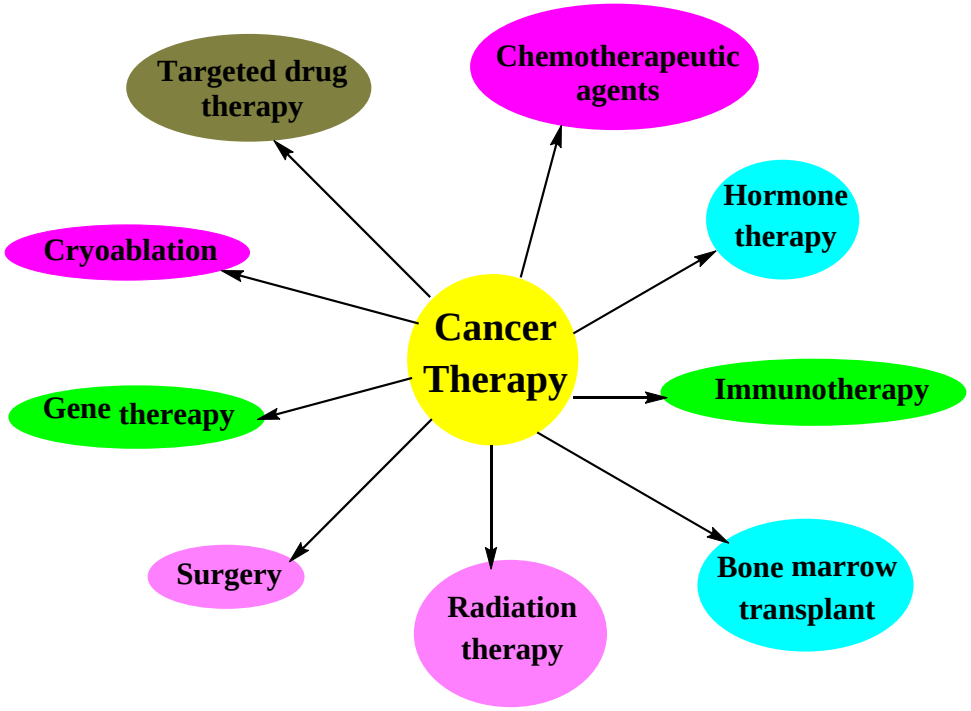


Figure 1.3: Available treatments against cancer

Among all the available treatments, chemotherapeutic agents are widely used as they have ability to arrest the growth of tumor cells. Many drugs are available which are used in chemotherapy which acts through different-different mechanisms. Although these agents have revealed good potency against cancer, still they are associated with several complications and issues. These include lack of selectivity, toxicity and many side effects (Huang *et al.*, 2017; Nurgali *et al.*, 2018). Major side effects associated with toxicity of chemotherapeutic agents include:

- Bone marrow toxicity
- Alopecia
- Inhibition of epithelial replenishment
- Diarrhoea
- Leucopoenia
- Impaired healing
- Teratogenicity
- Damage of germinal cells
- Thrombocytopenia
- Anaemia
- Unregulated erythropoiesis

- Halted multiplication of lymphocytes

1.1.4 Common targets for chemotherapeutic agents

It has been noticed that various chemotherapeutic agents possess anticancer activity by binding to a variety of targets (Bhatia *et al.*, 2019). Some of them have been mentioned below:

- Protein Kinase
- Aromatase
- Sulphatase
- TNF-α
- HSP90
- Topoisomerase
- Histone deacetylases
- Apoptotic factors
- Antimetabolites
- Tubulin polymerase
- Epidermal growth factor (EGF)
- Vascular endothelial growth factor (VEGF)

1.2 Breast cancer

Breast cancer refers to the development of a malignant tumour in breast cells (Hsiao *et al.*, 2010) Normal cells divide in a regulatory manner as per requirement. The cells become cancerous when they lose their ability to stop dividing, to attach to other cells, to stay where they belong, and to die at the proper time (Becker *et al.*, 2015). Breast cancer develops due to genetic abnormalities or mutations in genetic material in 85-90% cases whereas only 5-10% is inherited (Clezardin *et al.*, 2011). It involves the development of heterogeneous, morphologically distinct lesions with different biochemical and molecular characters (Ravnan *et al.*, 2011). Breast cancer is the prominent cause of mortality in women belonging to western countries and according to reports, it has been revealed that about one-third cases of post-menopausal breast cancer involved the over expression of oestrogen, leading to the development of hormone-dependent tumours (Cardoso *et al.*, 2010). According to the American Chemical Society (ACS), up to the end of 2017, over 250,000 new cases of invasive

breast cancer were diagnosed and almost 40,000 women have died due to breast cancer in 2015 (Bhatia *et al.,* 2019). It has been estimated that there will be 276,480 expected new cases of invasive breast cancer in U.S. women whereas 48,530 expected cases of non-invasive breast cancer. About 2,620 new cases of invasive breast cancer are expected to be diagnosed in men in 2020. A man's lifetime risk of breast cancer is about 1 in 883 (Yalaza *et al.,* 2016). Continuous efforts have been made towards developing newer medicines for the treatment of breast cancer with a good success rate. Despite advancement in the development of cytotoxics, chemotherapies, endocrine therapies, molecular inhibitors, and biologicals, metastatic breast cancer is still a major cause of death in postmenopausal women which suggests the requirement of new therapeutic candidates and drug combination therapies which will selectively focus on specific targets to cure the ailment (Chen *et al.,* 2008; Musa *et al.,* 2008; Moyer *et al.,* 1997; Lewis *et al.,* 2005; Harada *et al.,* 2010).

1.2.1 Types of breast cancer

Breast cancer is not a single disease; instead it has many types and subtypes collectively referred to as breast cancer. Broadly it can be classified into two types: Non-invasive and invasive cancers (Sharma *et al.,* 2010).

1.2.1.1 Non-invasive breast cancer

These are the types of breast cancer which occur in milk ducts or lobules of the breasts and do not invade normal breast tissues. These are regarded as carcinoma and also termed as pre-cancers. Non-invasive cancers are following types:

a. Ductal carcinoma in situ (DCIS)

It is the common form of non-invasive breast cancer starting in milk ducts of breast and do not spread to breast tissues. It is not lethal but there is maximum risk of developing an invasive cancer at later stages.

b. Lobular carcinoma in situ (LCIS)

This type of cancer develops in lobules (milk producing glands). It is also non-invasive and also not life-threatening.

1.2.1.2 Invasive breast cancer

Invasive breast cancers invade and spread into the surrounding breast tissues located outside the ducts and lobules. It may further involve lymph nodes in the breast and armpit but not other parts of the body. These cancers have been classified into following types:

a. Invasive ductal carcinoma (IDC)

It is the most frequent type of breast cancer and almost 80% cases of breast cancers involve IDC. It begins in milk ducts and got spread into surrounding tissues after breaking of milk duct lining. Later, it spreads into the lymph nodes and other parts of the body.

b. Invasive lobular carcinoma (ILC)

It is the second most frequent type of breast cancer after IDC involving milk-producing lobules of the breast and spread into surrounding tissue. Later, it also spreads into lymph nodes and other parts of the body.

c. Paget's disease of the nipple

It is a rare type of breast cancer which involves cells of nipple or the areola leading to scaly, red, itchy and irritated area. Patients suffering with this disease are also associated with DCIS or invasive breast cancers. Unusual transformations in the nipple and surrounding area are the 1st symptoms of Paget's disease.

d. Inflammatory breast cancer

It is rare form of invasive breast cancer affecting the blood vessels of the skin or lymphatic vessels in the breast leading to reddish and inflamed breasts.

e. Phyllodes breast tumors

These are rare and may be benign or malignant. These grow quickly but do not/rarely spread outside the breast. These develop in the connective tissues outside the ducts or lobules of the breast.

f. Advanced local breast cancer

It is large type of invasive cancer which has ability to spread outside the breast to the surroundings such as skin, chest or muscles and sometimes may also involve local lymph nodes.

g. Metastatic breast cancer

It is the advanced form of breast cancer generally termed as secondary or stage 4 breast cancer which has been spread to other body parts such as bones, liver and lungs.

1.2.2 Role of estrogens in breast cancer

It has been estimated that about 66% postmenopausal women suffering from breast cancer are associated with estrogen dependent breast cancer. Estrogen binds to the estrogen receptors (present in mammary glands) and promotes the tumour growth in female breasts (Brisken *et al.,* 2010). Estrogens cause enhancement of proliferation of breast epithelial cells and estrogen dependent mammary carcinoma cells and secrete various growth factors (Arendt *et al.,* 2015). Estrogens have two receptors for binding namely estrogen receptor (ER)-α and ER-β. ER-α was identified at very first and maximum reports related to estrogen activity are on the basis of impact of ER-α in the mammary gland. Later the presence of ER-β has been also confirmed but its pharmacological functions are still under investigation. ER-α performs its function as transcription factor which is activated by the ligands. Biological effects of estrogens are exhibited by interaction with estrogen-response elements present in specific genes followed by gene expression (Wang *et al.*, 2010). Other extracellular stimuli also have tendency to activate ER-α-mediated transcription if estrogen is absent. It is evident that ER-α can influence gene expression without directly binding to the DNA (Zhang *et al.,* 2002). A few studies have also postulated that estrogens can also exert non-genomic mechanism of action through interaction with other proteins like a putative membrane estrogen receptor, growth factor receptors, and intermediate cell signalling molecules. Cancers have been classified clinically as ER-α-positive or ER-α-negative. While patients with ER-α-positive tumors have a somewhat superior endurance rate than ER-α-negative patients, expression of ER-α is a significant possible factor for response to endocrine therapy. Defects in estrogen receptors (ERs) and insulin resistance are two inter-related pathologic conditions which may lead to development of breast cancer (Duss *et al.,* 2007).

One of the predominant mechanisms of breast cancer progression among woman is insulin resistance (Arcidiacono *et al.,* 2012; Eketunde *et al.*, 2020). It was worth notable that in maximum of breast cancer patients, insulin resistance was

accompanied with hyperandrogenism, disordered menstrual cycle, anovulation and complex endocrine complications in the environment of faulty glucose uptake. It is basically a defect in insulin mediated cellular glucose uptake which is associated with defected gene expression responsible for metabolism, growth, differentiation and proliferation (Poloz *et al.,* 2015). This disorder imposes a patient into a variety of disorders like type 2 diabetes mellitus, malignancies, metabolic disorders, cardiovascular lesions etc. Hyperinsulinism activates the refractory insulin receptors, insulin-like growth factors such as IGF-1 which are further responsible alterations in cell proliferation as well as tumor growth (Durrani *et al.,* 2021). Overproduction of insulin is also responsible for disturbances in endocrine discharges. Hyperinsulinism shifts the production of estrogens towards androgens by inhibiting the ovarian as well as adrenal aromatase. Also insulin can promote secretion of luteinizing hormone of the pituitary gland resulting into increased androgen production. This accumulation of androgens and decreased production of estrogens is harmful for the normal cellular mechanisms (Ormazabul *et al.,* 2018).

Another prominent mechanism associated with breast cancer is defects in estrogen receptors (ERs) (Shao *et al.,* 2003). Lack or decrease in ER reactivity leads to excess of estrogen synthesis followed by a feedback mechanism resulting into hyperestrogenism. Estrogen levels may also be enhanced without appearance of any symptom, in women having BRCA gene mutations. These women bear a lifelong enhanced risk for development of breast cancer. Estrogens are responsible for cancer in two ways. It enhances the cell divisions in breast tissue by acting as a mitogen leading to defects in cell division (Bhardwaj *et al.,* 2019). Also, its metabolites act as carcinogens or genotoxins causing damages to DNA leading to generation of cancerous cells. A few environmental estrogens line xenoestrogens or phytoestrogens can also be exposed as they work similarly to human estrogens. Xenoestrogens have structural similarity to human estrogens and has the ability to bind to the estrogen receptors. Excess activity of estrogen may also stimulate hormones like relaxin which potentiate cell division as well growth and differentiation (Burns *et al.,* 2012). All these facts have been evidenced by several studies that there is a direct relationship between endogenous hormone levels in postmenopausal women and risk of breast cancer. Figure 1.4 outlines the major manifestations associated with estrogen receptor defects, insulin resistance and their role in breast cancer development.

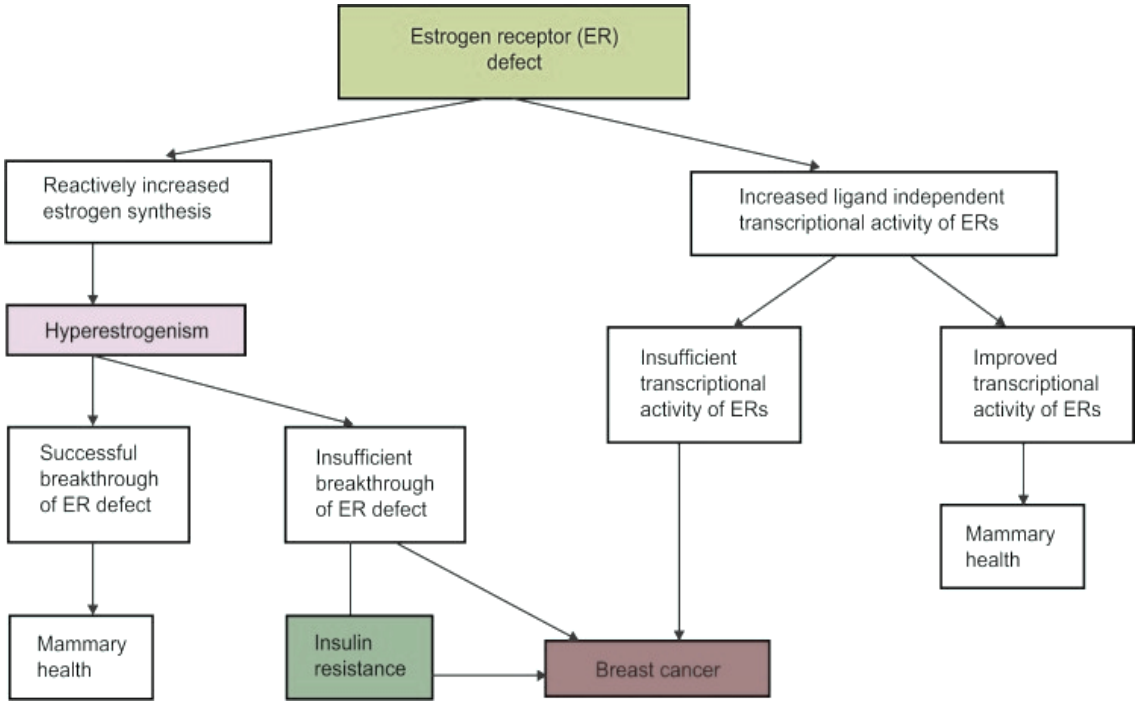


Figure 1.4: Impact of estrogen in development of breast cancer

1.2.3 Role of aromatase and its inhibitors

It has been well established that in estrogen-dependent breast cancer, estrogens express peptide growth receptors which cause proliferation of the cancer cells. Aromatase belongs to cytochrome P450 enzyme class which is present in ovaries of premenopausal, adipose tissue of postmenopausal and placenta of pregnant women in higher concentrations (Gao *et al.*, 2020). It is evident that aromatase is highly expressed in tumor sites of breasts (Brueggemeier *et al.*, 2019; Zhao *et al.*, 2016; Kristensen *et al.*, 2000). Aromatase synthesizes estrogens by converting C-19 androgens to C-18 estrogenic steroids through three hydroxylation reactions. It converts androstenedione to estrone and testosterone to estradiol (Harbeck *et al.*, 2019) (Figure 1.5). Hence abnormal expression of aromatase in breast cells or adipose stromal cells specifically in postmenopausal women has significant effect on development and progression of breast tumors (Bollet *et al.*, 2009). Therefore aromatase has been identified as a significant target for development of therapeutic agents against breast cancer. There are many aromatase inhibitor drugs available in market against breast cancer. Aromatase inhibitors have been classified into two major classes: Steroidal (Exemestane, Formestane, Testolactone) and non-steroidal

(Anastrozole, Letrozole, Vorozole, Aminoglutethimide) aromatase inhibitors (Figure 1.6).

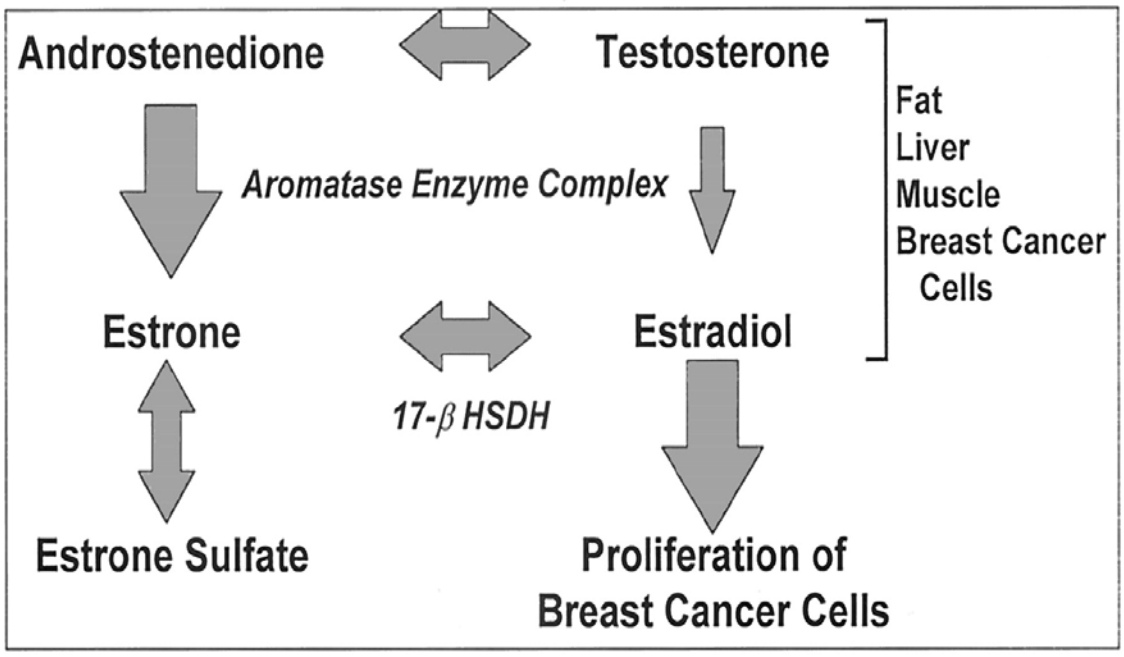


Figure 1.5: Role of aromatase expression in breast cancer progression

It is worth notable that aromatase inhibitors (AIs) have been proved to be the preferred choice for treatment of breast cancer in postmenopausal women. They have revealed excellent clinical benefits and reduced mortality associated with breast cancers. A few clinical evidences have raised an issue regarding their safety by reporting an increased risk of cardiovascular complications associated with them. But the clear pathological mechanism is still unclear and is under investigation. Some randomized controlled trials results have reported certain events of hypercholesterolemia also. The first generation aromatase inhibitor (AI) aminoglutethimide was found associated with sedative side effects as well as adverse effects on central nervous system (Robert *et al.,* 2005). Second generation AIs formestane and fadrozole were found well tolerated but potency was less than the tamoxifen drug. Later on exemestane was discovered which was found highly potent and selective which acted by interfering with the binding sites of the enzyme and form irreversible bonding with the enzyme (Ferratti *et al.,* 2006). Other non-steroidal AIs like anastrazole and letrozole block electron transport chain by acting as competitive inhibitors. Common side-effects related to AIs include hot flushes, gynecologic events, skeletal problems, sexual dysfunction, cardiac complications, neurotoxicity

and other organs toxicity (Bhatnagar *et al.,* 2007). Therefore development of newer class of anti-breast cancer agents having potentials to inhibit aromatase is the great need of the hour so as to bypass the complications associated with traditional therapeutic candidates.

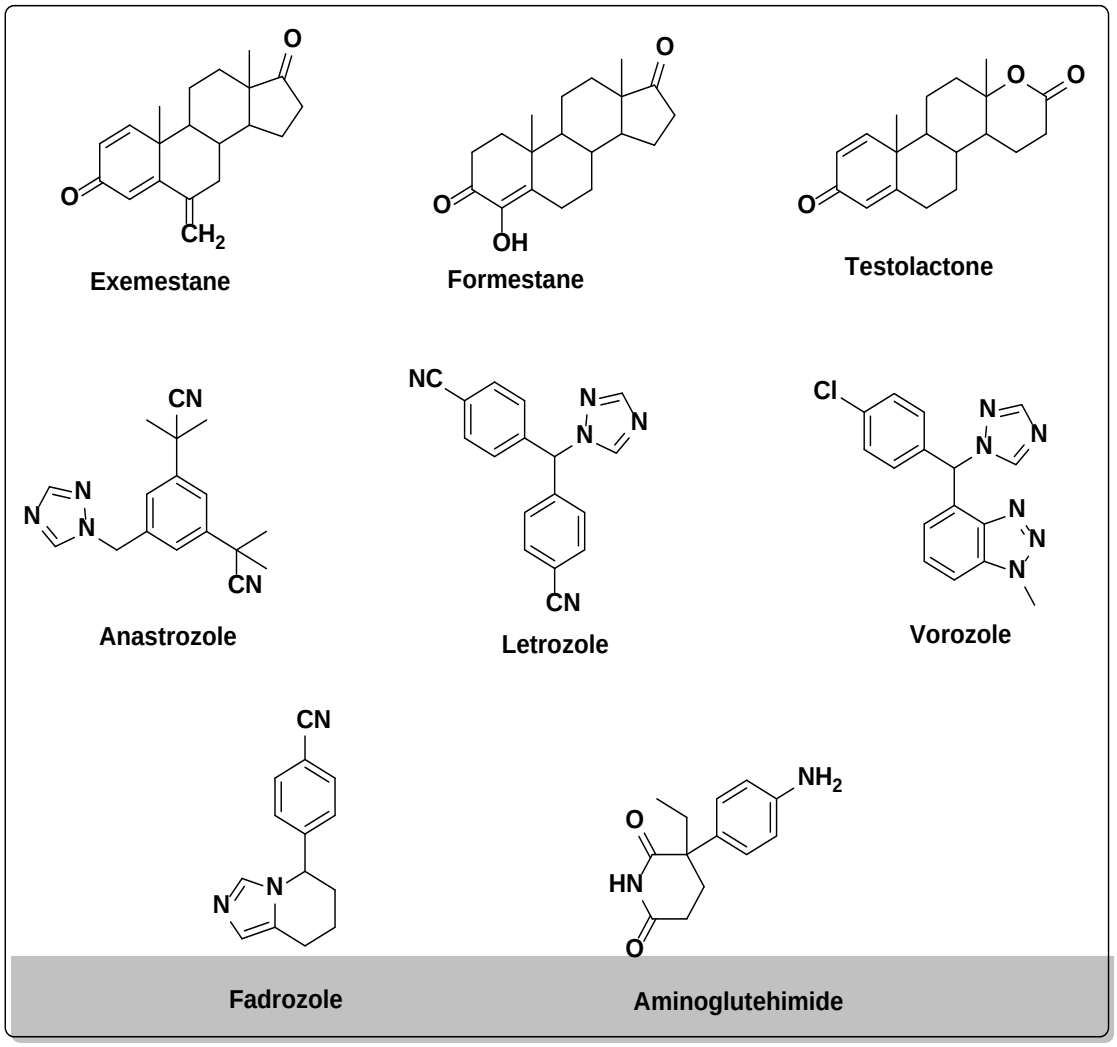


Figure 1.6: Steroidal and non-steroidal aromatase inhibitors

1.2.4 Role of human epidermal growth receptor (HER2)

Human epidermal growth receptor (HER2) plays a significant role in development and progression of breast cancer (Iqbal *et al.,* 2014). It consists of 1255 amino acids, having molecular weight of 185 kD belonging to transmembrane glycoproteins located on long arm of human chromosome 17. It is expressed in several tissues which are responsible for rapid growth of the cells. HER2 gene produces HER2 proteins which are the receptors present on breast cells (Gutierrez *et al.,* 2011). HER2 receptors regulate normal breast cells growth, division and repair. The HER2

receptors are present on the cell surface as monomers which undergo dimerization and transphosphorylation upon ligand binding to their extracellular domains (Moasser *et al.,* 2007). But it is evident that in almost 15-20% cases of breast cancers, the HER2 gene gets defected and makes many copies of it called as process of HER2 amplification. These extra HER2 genes direct the breast cells to produce many copies of HER2 leading to protein over-expression (Figure 1.7). All these events result into uncontrolled division and growth of breast cells. Breast cancers occurring due to HER2 amplification and HER2 protein over-expression are regarded as HER2-positive (Schlam *et al.,* 2021). These cancers grow and spread rapidly as compared to other HER-2 negative breast cancers.

Thus, HER2 has been identified as a promising target for design and develop newer therapeutic agents against breast cancer. A few drugs are already available in market as a targeted drug therapy for inhibition of HER2. Targeted cancer therapies target characteristic features of cancer cells like some proteins which are responsible for rapid division/growth of cancer cells in abnormal way. Targeted therapies are more selective and do negligible or very less harm to normal and healthy cells. The available drugs include monoclonal antibodies such as trastuzumab, pertuzumab, hyaluronidase and margetuximab (Nahta *et al.,* 2006). These monoclonal antibodies get attached to the surface of HER2 protein of cancer cells leading to stop the growth of these cells. Trastuzumab is a prominent therapeutic candidate which can be used in the treatment of early stage as well as advanced stages of breast cancer. Other treatments include use of antibody-drug conjugates in which a monoclonal antibody is linked with a chemotherapeutic drug. In this mechanism, the antibody acts as governing channel by adhering to the HER2 protein on cancer cells and bring the chemotherapeutic agent to them. Examples of such a treatment include ado-trastuzumab emtansine and fam-trastuzumab deruxtecan. Alternatively kinase inhibitors like lapatinib, neratinib and tucanitib can also be used to inhibit the actions of HER2.

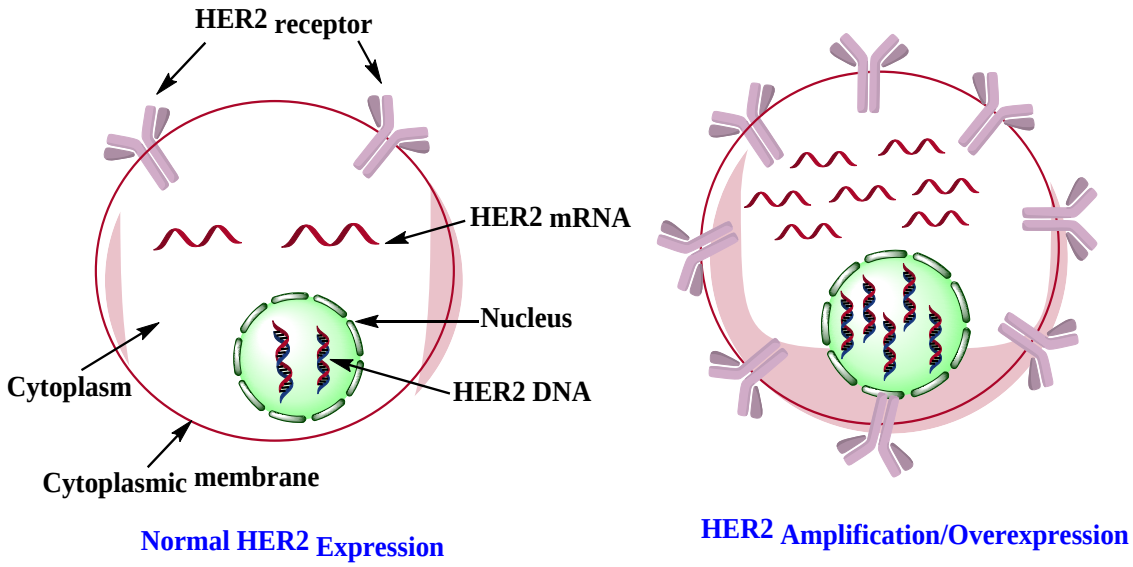


Figure 1.7: Consequences of HER2 over-expression

1.3 Coumarin

Coumarin nucleus is one of the most potent heterocyclic scaffolds. It belongs to the benzopyrone chemical class possessing a wide range of biological activities. There are several coumarin nucleus containing drugs available in market as potent medicines. Coumarin and its derivatives have been found to exhibit very rare nephrotoxicity, hepatotoxicity, cardiotoxicity, dermal toxicity and other side effects. Coumarin (2*H*-1-benzopyran-2-one) finds its origin from plants consisting of a class of phenolic substances and is constructed through fusion of benzene and α-pyrone rings (Figure 1.8) (Venugopala *et al.,* 2013). Approximately 1300 coumarins have been recognized as secondary metabolites from plants, bacterial and fungal sources. 1st coumarin was isolated in 1820 from plant source. A total of about 150 species of coumarins have been distributed among more than 30 plant families including Umbelliferae, Clusiaceae, Guttiferae, Oleaceae and Rutaceae (Stringlis *et al.*, 2019; Sarker *et al.*, 2017). Coumarin derivatives have been also found in essential oils like cinnamon bark oil, cassia leaf oil as well as in some fruits, green tea and food stuffs. Coumarin possesses diverse pharmacological activities like anticoagulant, anti-HIV, antibacterial, antioxidant, antihypertensive, anti-tubercular, anticonvulsant, antifungal, anti-hyperglycemic, scavenging of reactive oxygen species (ROS), anti-inflammatory and anticancer (Figure 1.9) (Wu *et al.*, 2009; Srikrishna *et al.;* 2018). A few bioactive coumarin based drugs have been depicted in Figure 1.10. Anti-cancer activity is the

most important among them and researchers have nowadays paid keen attention to exploring this potency of coumarin nucleus to develop novel therapeutic agents in the treatment of breast cancer. It has been reported that coumarin nucleus possesses activity against cancer by binding to the various biological targets such as aromatase, sulphatase, protein kinase, selective estrogen receptor modulator (SERM), selective estrogen receptor down regulator and 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3) inhibitor (Bhatia *et al.*, 2019). Coumarin scaffold exists as simple substituted compounds as well as complex hybrid forms due to its structural diversity. By focussing on these characteristics of the coumarin scaffold, many different pharmacophores can be combined with coumarin motif to design coumarin hybrids.

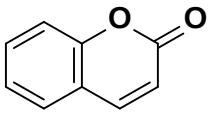


Figure 1.8: Structure of coumarin

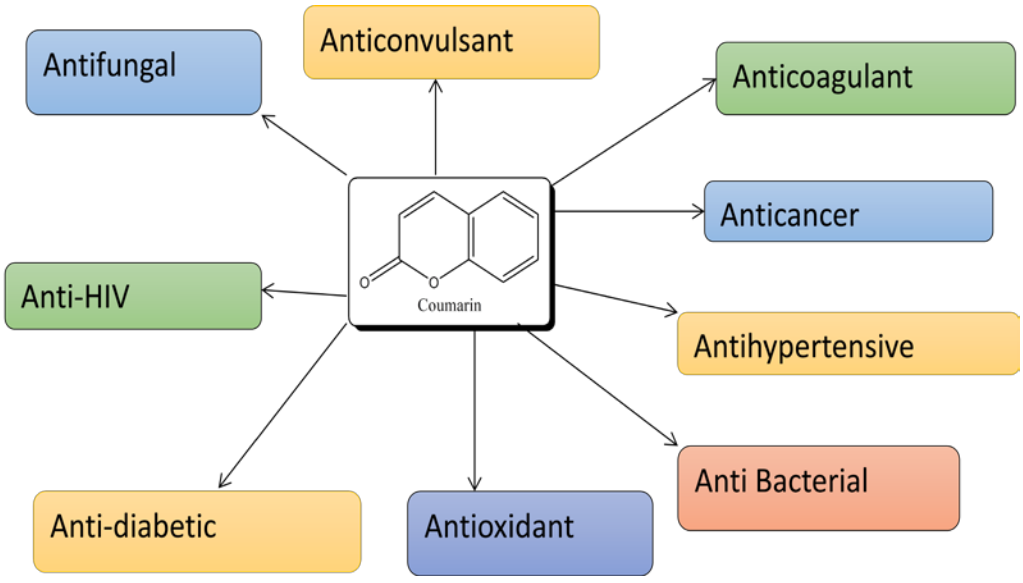


Figure 1.9: Pharmacological diversity of coumarins

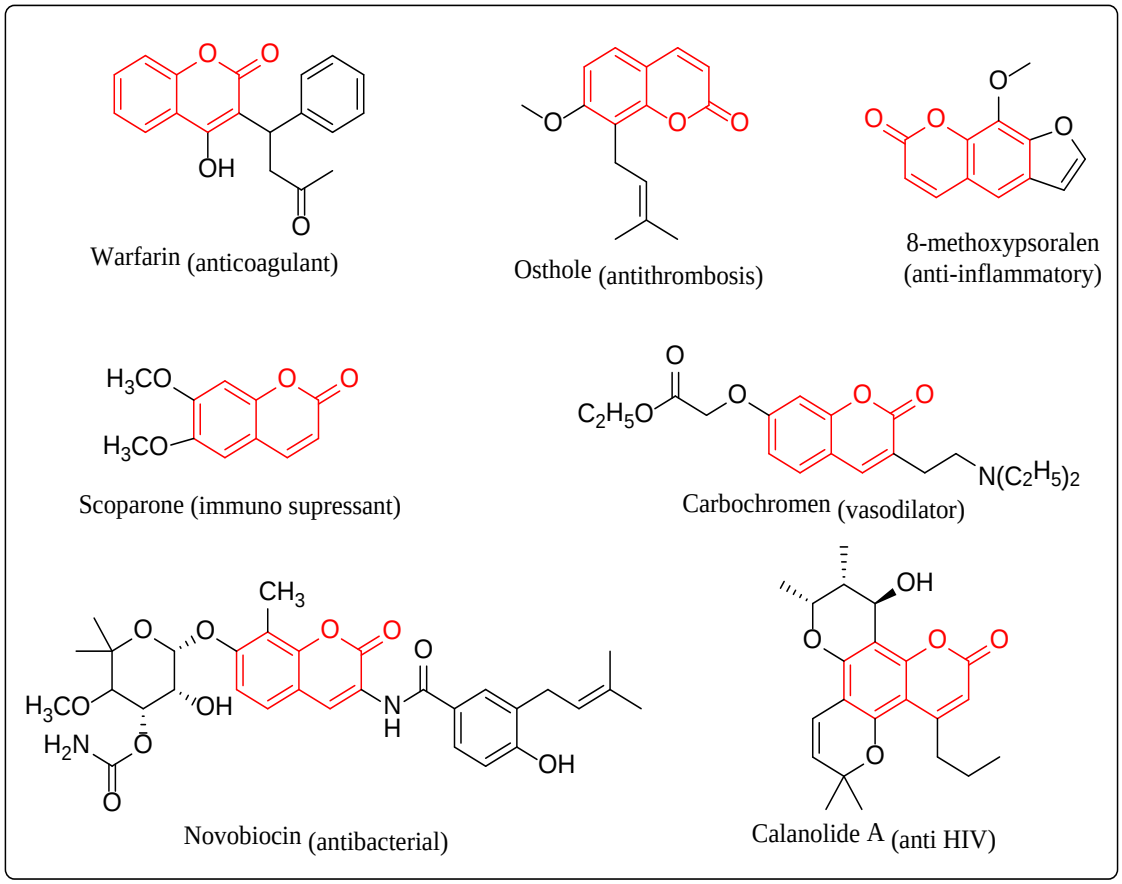


Figure 1.10: Some bio-active coumarin containing drugs

1.4 Concept of coumarin hybrid molecules

In the past decade, the combination therapies have been evolved as promising tools in the development of newer anticancer drugs by utilizing the approach of molecular hybridization. This approach is based upon creation of new ligands by combining two or more existing motifs/sub-unit of motif through suitable chemical approach leading to generation of hybrid molecules which have all the properties of original motifs (Adriano *et al.*, 2009). Thus the formulated hybrids have ability to interact with two or more than two targets and provide improved activity as compared to single motif (Mishra *et al.*, 2016). The different pharmacophores in the hybrid molecules may possess different modes of action based upon the particular target to which they interact (Ballazhi *et al.*, 2015). Figure 1.11 depicts the basic concept and motive of generation of hybrid molecules. Coumarin hybrids can be constructed by following the same strategy by combining it with different bioactive scaffolds with specific mode of action through suitable sites. Thus coumarin hybrids are capable of binding to different targets and to provide excellent pharmacological activity against cancer. A few reported multitarget-directed coumarin hybrids have been depicted in Figure 1.12.

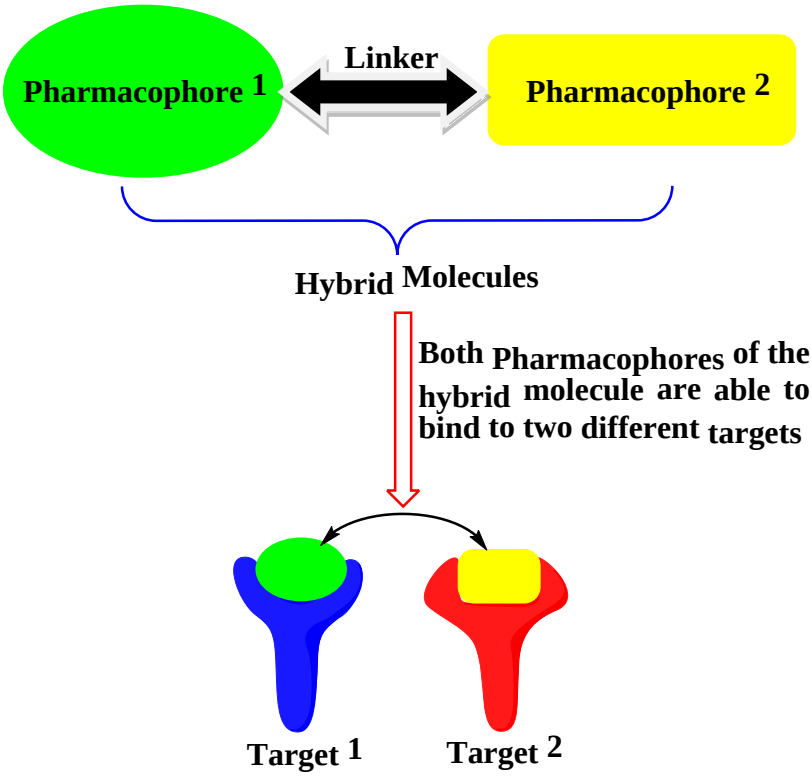


Figure 1.11: Concept of molecular hybridization

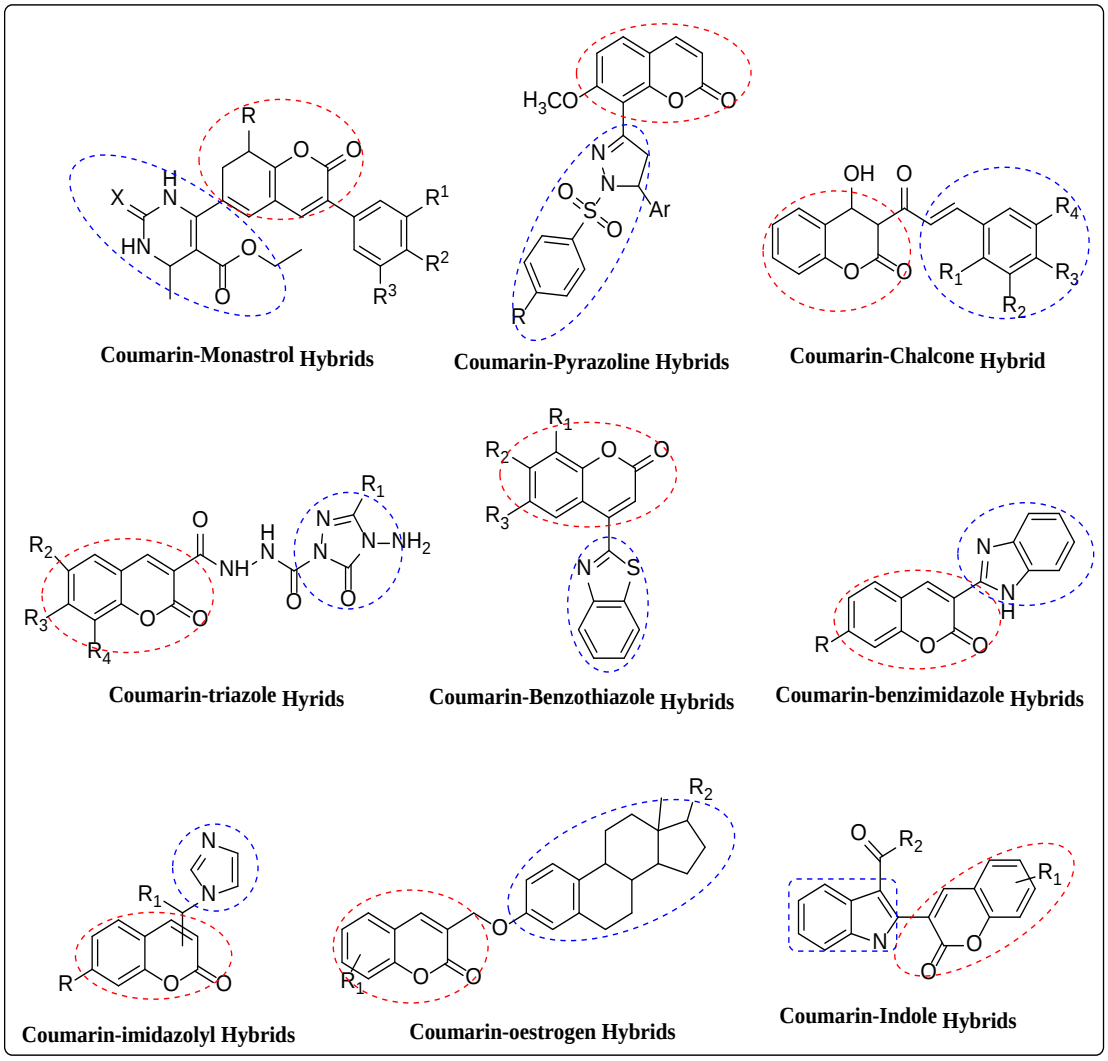


Figure 1.12: Some coumarin hybrid compounds against breast cancer

1.5 Selected pharmacophores for hybridization with coumarin

1.5.1 Quinoxaline

Quinoxaline is a heterocyclic compound comprising fusion of benzene and a pyrazine ring (Figure 1.13). Quinoxaline is another potent scaffold which has been explored by several research groups for its anti-cancer potentials. Many reports are available in literature revealing its promising activity against breast cancer (Newahie *et al.*, 2019; Bayoumi *et al.*, 2019). Quinoxaline exhibits its anti-cancer potentials by inhibiting human protein kinases which are responsible for cell proliferation, migration and differentiation (Newahie *et al.*, 2016; Unzue *et al.*, 2014; Kim *et al.*, 2020; Alswah *et al.*, 2018; Sibiya *et al.*, 2019). Qunoxaline derivatives also stimulate production of reactive oxygen species (ROS) resulting into cell apoptosis and cytotoxicity.

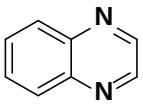


Figure 1.13: Structure of quinoxaline

1.5.2 Dihydropyrimidinone

Dihydropyrimidinones are heterocycles with a pyrimidine moiety (Figure 1.14) in the ring nucleus, which, in recent decades, have raised great attention in medicinal chemistry due to versatile biological activity. One of the prominent activities of dihydropyrimidinone (DHPM) is the anti-cancer activity which has been continuously explored by several research groups (Kaur *et al.,* 2017). Also, this class of compounds causes mitotic arrest at G2/M phase by blocking bipolar mitotic spindle in mammalian cells leading to cell apoptosis. There are several reports has been available in literature which give success story of dihydropyrimidinone derivatives as cytotoxic agents (Bhat *et al.,* 2016; Amany *et al.,* 2018; Reddy *et al.,* 2013; Soumyanarayanan *et al.,* 2012). Also these have potentials to stimulate production of ROS which result in cancer cell apoptosis.

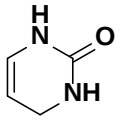


Figure 1.14: Structure of dihydropyrimidinone

1.5.3 Dihydropyridine

Dihydropyridine (DHP) is a nitrogen containing heterocycle based upon pyridine (Figure 1.15) and belongs to a chemical class of molecules that is semi-saturated with two substituents replacing one double bond. DHP derivatives keep a significant place in pharmacology as they have tendency to block L-type calcium channel blockers and they are integral part of the structure of several anti-hypertensive agents. Beyond this, DHPs have been also found to exhibit significant anti-cancer activity and are continuously explored to develop newer therapeutic agents against cancer (Viradiya *et al.,* 2017; Mohamed *et al.,* 2018; Sayed *et al.,* 20121; Vanhoefer *et al.,* 1999). They reveal anticancer potentials by inhibiting topoisomerase, aromatase, sulphatase and protein kinase. Also they directly inhibit apoptosis and oxidative stress caused by reactive oxygen species.

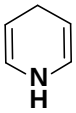


Figure 1.15: Structure of dihydropyridine

1.6 Molecular docking

Molecular docking is referred to a computer simulation program used to predict the possible confirmations of a ligand-receptor complex. A receptor is a protein target or a nucleic acid molecule (DNA or RNA) and a ligand is a drug or small molecule or any other protein. This investigation is then utilized to know about preferred orientation of the ligands and to predict the binding affinity of small molecules. Docking simulations are useful in knowing the position of ligand in the binding pocket of the protein or receptor. 3D structure of a target protein is required to run a docking protocol which can be a X-ray crystal structure or a homology model. Docking can also be used for validating a protein model as well as to identify the significant amino acids which have been involved in ligand-protein interactions. It is a promising approach which has been widely adapted in structure-based drug design due to its ability to find out the binding patterns of a molecule with the target. Hence it has been successfully applied in rational drug design against various ailments (Morris *et al,* 2008; Meng *et al.,* 2011).

The prime aim of molecular docking is to predict drug-receptor binding patterns utilizing computational programs. Docking can be run into two steps: Firstly confirmations are generated in binding pocket of the protein and secondly ranking of these confirmations is done by applying some scoring functions to them. It has been proven to be a key methodology nowadays in structural molecular biology and computer-aided drug designing. Docking can be utilized to carry out virtual screening of large molecular libraries, to rank the outcomes on the basis of scoring functions, to predict the target interaction mode as well as to identify a lead molecule. A typical docking protocol involves following steps (Figure 1.16):

a. Selection and preparation of target

The target structure can be selected on experimental basis as well as it can be procured from X-ray crystallographic data. The structures of targets can be downloaded from protein data bank or a homology model can be generated for

docking. Other online databases like MOAD also provide structures of targets which are suitable for docking. The procured target from the databases is generally not flexible and a target can attain several confirmations or structural changes on binding of a particular ligand to it. To solve this problem, a target needs to be prepared before subjecting to the docking process. Molecular dynamics program have several features to prepare a ligand which include target flexibility, effect of presence of solvents, induced fitness etc. Various steps in target preparation include selection and deletion of internal ligands, selection of appropriate forcefield, isolation of atoms, selection of amino acid chains, creation of dummies and labelling etc.

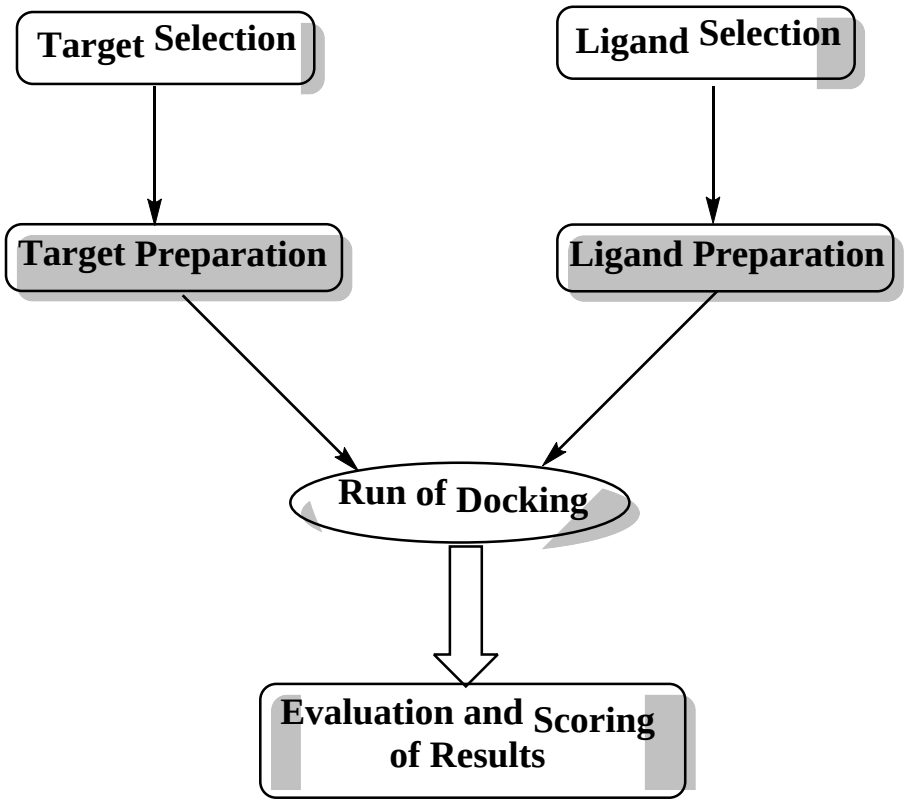


Figure 1.16: Steps involved in a docking procedure

b. Selection and preparation of ligands

The selection of ligands depends upon the type of investigation and several filters are available in computer program e.g. for lead discovery several features are available such as net charge, molecular weight, polar surface area, availability, solubility etc. can be selected. For lead optimization purpose filters like similarity thresholds, pharmacophores, synthetic accessibility, and

pharmacokinetic/toxicokinetics properties can be applied. The 2D structures of designed compounds are generally drawn in Chemdraw Professional software and saved as mol files. Then suitable forcefield, algorithm scoring gradient is selected followed by the process of energy minimization. Prepared ligands are then saved in a compatible formal such as .mol, .pdb, .mdb etc.

c. Run of docking protocol

Docking calculations are then performed using suitable software. A grid is generally prepared with the center defined by the co-crystallized ligand. Validation of docking program is done by reproducing the conformation of co-crystallized ligand positional space of the Docked ligand followed by elimination of unwanted conformations and finally calculation of root mean square deviation (RMSD) between co-crystallized and docked conformation is done. Positional space of the docked ligand and unwanted conformations is eliminated. Firstly, various conformations of ligand inside receptor pocket are generated by the software using genetic algorithm based search function. For this analysis, approximately 100000 operations are carried out on a population size of 100 and crossover, mutation and migration frequencies are set to default values of 95, 95 and 10 respectively. Secondly, ranking of various conformations are done using various scoring functions. On the basis of scores, the best conformations are chosen for interaction study with crucial residues of binding cavity. The interactions of these ligands are then thoroughly studied to estimate their binding affinities, hence biological activities.

1.7 Common docking softwares

There are many docking softwares available with wide variety of advanced functions (Pagadala *et al.*, 2017; Fan *et al.*, 2019). A few of them have been listed below:

- Schrodinger
- MOE
- GOLD
- Autodock
- PyRx
- Darwin

- Glide
- QXP
- FleX
- WISDOM
- LUDI
- SMOG
- VLife

1.8 Research Envisaged

Although many efforts for treatment of cancer diseases have been carried out and much progress have been eventuated from diagnosis to treatment of cancer, but some of cancer patients do not respond to therapy or recurrence subsequent initial response. Nevertheless, chemotherapy is a basic approach for the treatment of cancer diseases. One of the most important obstacles in chemotherapy is drug resistance to many anticancer agents (Mansoori *et al.,* 2017). Drug-induced toxicities followed by administrating high doses of chemotherapeutic agents to overcome drug resistance were arisen. Accordingly, the discovery of new anticancer agent with promising activity and high therapeutic index is an urgent need (Housman *et al.,* 2014). Many researchers have explored various heterocyclic compounds to get maximum therapeutic efficacy from them. With a vision of novel drug design, the concept of molecular hybridisation is a significant approach which involves combination of two structurally diverse motifs in a single molecule with excellent therapeutic potential.

It is well evident from literature that coumarin scaffold possesses significant anticancer activity and also has aromatase inhibition potential (Yamaguchi *et al.,* 2017; Chen *et al.,* 2004; Musa *et al*., 2008; Stefanchi *et al.,* 2011; Yamaguchi *et al.,* 2020). There are so many coumarin derivatives already have been synthesized as potent anti-cancer agents. But maximum synthetic strategies involve complex processes, yield problems and sometimes very low potency is observed. So far, there is no official coumarin derivative drug candidate has been reported for cancer therapy. On the other hand, there is a toxicity issue in the existing anti-cancer drugs. To overcome these problems we have intended to explore anti-cancer activity of coumarin nucleus as it causes very rare nephrotoxicity, neurotoxicity, cardiac toxicity and dermotoxicity. In the presented research work we have combined drug design

strategies with the new synthetic procedures. We have firstly carried out docking studies upon the proposed coumarin derivatives so as to get a preliminary idea about the predicted biological activity of the derivatives against anti-cancer targets. After getting the *in silico* information about the interactions of proposed compounds with the suitable anticancer target, we have synthesized the proposed compounds who displayed maximum docking scores and interactions. The synthesized compounds were further screened out for their *in vitro* anticancer activity.

1.8.1 Rationale for the designed work

Considering the versatility and structural features of coumarin, three series of coumarin fused nitrogen contatining heterocyclic derivatives were designed. The moieties selected for attachment with coumarin were quinoxaline, dihydropyrimidinone and dihydropyridine. These moieties were chosen after a thorough literature survey by knowing their anticancer potentials. The designed molecules possess therapeutic potentials of two moieties in a single molecule. Coumarin can exhibit anti-cancer potentials by interacting with several targets like aromatase, sulphatase and protein kinase. Also coumarin has very low *in vivo* toxicity. Beyond this, quinoxalines, dihydropyrimidinones and dihydropyridines are well established moieties in medicinal chemistry for their anti-cancer potentials. They act as significant apoptosis inducers, kinase inhibitors, topoisomerase inhibitors, ROS stimulators and also capable of inducing tumor specific cytotoxicity. Figure 1.17, 1.18 and 1.19 has depicted the rationale behind design of three series of coumarin derivatives.

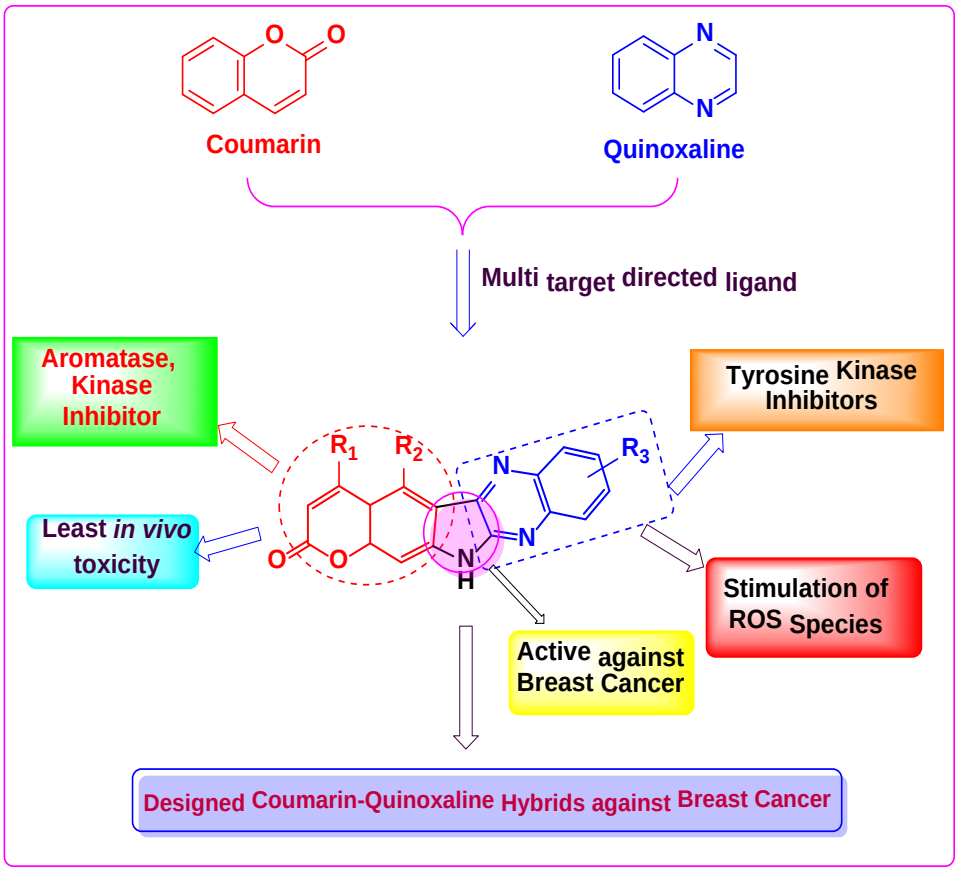


Figure 1.17: Rationale behind the design of coumarin-quinoxaline hybrids

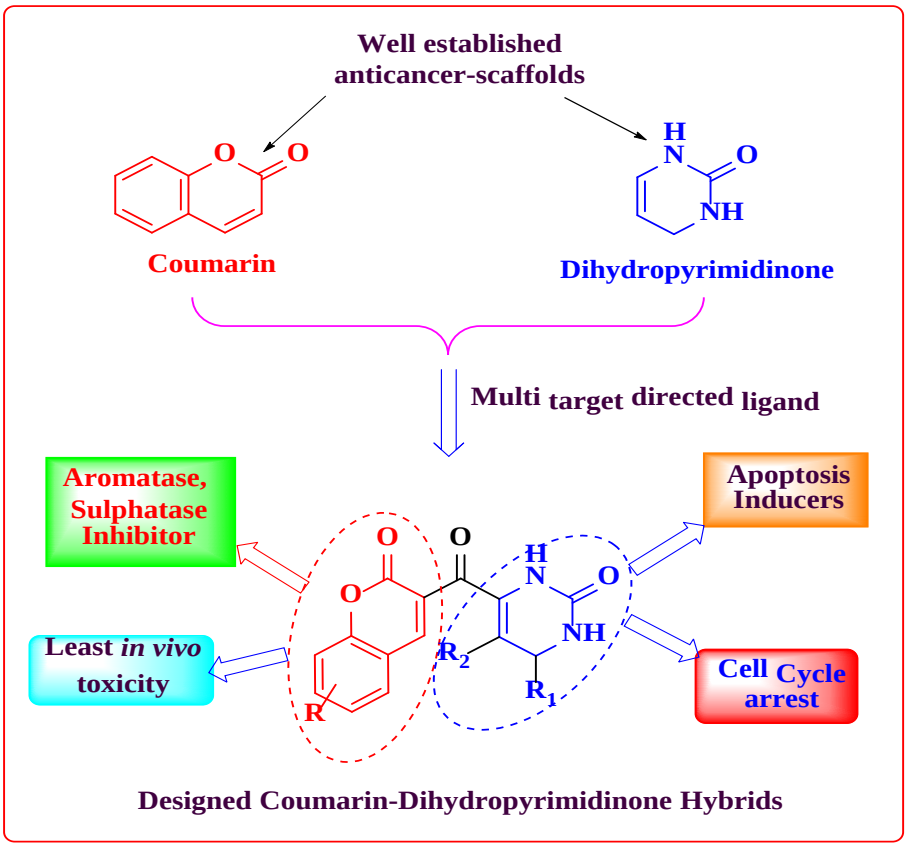


Figure 1.18: Design strategy for coumarin-dihydropyrimidinone hybrids

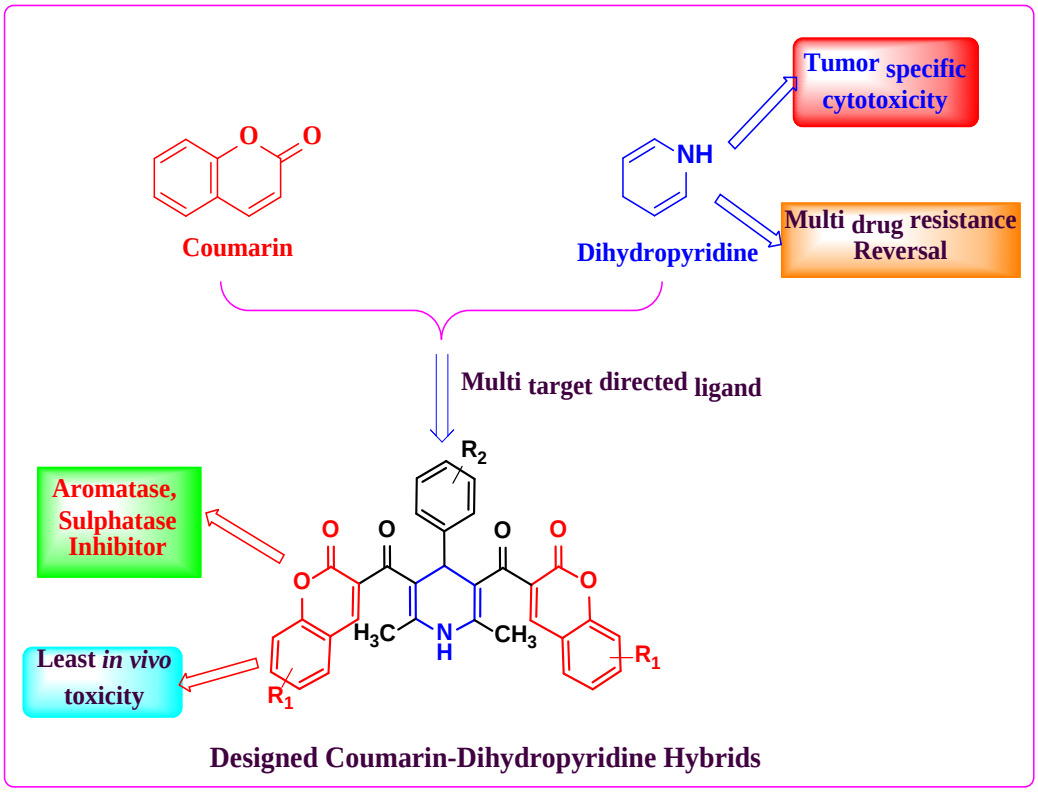


Figure 1.19: Design strategy for coumarin-dihydropyridine hybrids

1.9 Aim and Objectives

In the proposed work we have hypothesized fusion of various potent nitrogen containing scaffolds such as dihydropyridine, dihydropyrimidinone and quinoxaline with coumarin nucleus to form hybrid molecules to explore their cytotoxic potentials. The deigned research work was aimed to achieve the following objectives:

- To design coumarin tethered/fused nitrogen containing heterocycles based molecules and screen for activity against selected targets by molecular modelling tools.
- To synthesize and characterize the compounds predicted to be anticancer.
- To evaluate *in vitro* anticancer activity of the synthesized compounds against various cancer cell lines.
- To evaluate the active compounds for normal cell toxicity.

1.10 Plan of Work

The proposed research objectives were planned to be achieved in step wise step manner as presented hereunder:

A. Design of coumarin fused hybrid molecules

- Exhaustive literature survey on coumarins and their anti-cancer potentials
- Selection of nitrogen containing heterocycles to be fused with coumarin scaffold
- Identification of significant targets to design therapeutic candidates against cancer

B. Molecular docking studies

- Generation of libraries of different designed coumarin derivatives
- Selection of suitable anticancer targets
- Preparation of target
- Preparation of ligands
- Run of docking
- Assigning of scoring functions
- Validation of docking
- Analysis of binding patterns and interactions
- Procurement of 2D and 3D interaction poses
- Screening of potent compounds on the basis of docking outcomes

C. *In silico* studies on screened compounds

- *In silico* drug likeliness prediction
- *In silico* ADME prediction
- *In silico* toxicity prediction

D. Synthesis of best screened compounds after docking

- Selection of suitable synthetic strategies
- Procurement of chemicals and reagents
- Run of synthetic procedures
- TLC analysis and purification of synthesized compounds

E. Spectral characterization of synthesized compounds through

- ^{1}H-NMR

- ^{13}C-NMR
- Mass Spectrometry

F. Anticancer activity of synthesized compounds

- Selection of various cancer cell lines
- Evaluation of anti-cancer potentials on various cell lines through MTT assay
- Statistical treatment of the data

G. Evaluation of synthesized compounds for normal cell toxicity

H. Compilation of results and thesis writing