Chapter 1: Introduction

Contents

1.1 General facts about Cancer:

Cancer is a major cause of death worldwide. Cancer comes from a Greek word 'Karkinos' to describe carcinoma tumors by a physician Hippocrates (460-370 B.C). Evidence of human bone cancer is already found in Egyptian mummies. Although cancer can occur at all ages, it becomes more prevalent as people grow older. In recent times, there has been an increase in incidence of cancer in India as nearly two people are diagnosed every minute with cancer. This is mainly attributed to urbanization, industrialization, life style changes, population growth and increase in life span [1]. In India, life expectancy in 1971 was 45 years, then it rose to 62 years in 1991 and to 71 years expected by 2021-25 and so has the risk to harbor cancer. In short, the ultimate goal for cancer treatment still remains a distant hope. Because cancer cell closely resembles normal cells, which makes the treatment more challenging. In cancer treatment, the fundamental problem is to selectively kill cancer cells leaving normal cells un-affected [2].

The incidence of and mortality rate from cancer has become globally important. Chemotherapy using drugs that target cell division, angiogenesis or those who induce cancer cell death by various signaling pathways is one strategy to treat cancer. However, due to side effects and the development of drug resistance in cancer cells, there is needed to design, synthesize and develop more potent and safer chemotherapeutic agents.

The Indian Council of Medical Research (ICMR), New Delhi reports in 2016 that the total number of new cancer cases is expected to be around 14.5 lakh and the figure is likely to reach nearly 17.3 lakh new cases in 2020[3].

1.2 Normal cell versus Cancerous cell

Two prominent cancer scientists, Robert Weinberg and Douglas Hanahan have listed the hallmarks of Cancer, listing the eight very prominent traits of cancer cells [4]. They explained the eight biological capabilities which cell acquire in the process of transformation from a normal cell to cancerous cell. Table 1.1 gives the comparison between cellular activity occurring in normal cell and cancerous cell.

Table 1.1: Comparison of cellular activities in normal cell and cancerous cells

Hallmarks	Normal Cell	Cancerous Cell
Sustaining proliferative signaling	Grow only when stimulated by external signals	Stimulate their own growth.
Evading growth suppressors	Tumor-suppressor genes take care of the controlled cell division.	They disable tumor-suppressor genes and ignore external signals ordering them to stop dividing.
Resisting Cell death	Damaged and dangerous cells are destroyed by apoptosis.	They refuse to destroy themselves through apoptosis – the process of programmed cell death that rids the body of damaged and dangerous cells.
Enabling replicative immortality	Controlled cell division	Unlike normal cells, which can divide a limited number of times, cancer cells can multiply indefinitely, and are said to be "immortal."
Inducing angiogenesis	Controlled angiogenesis is observed.	They simulate the growth of new blood vessels (angiogenesis) to support tumors' increasing size.
Activating invasion and metastasis	These cells are localized and do not leave there surroundings that easily.	Cancer cells can break away from their site of origin, enabling them to invade surrounding local tissue and spread to distant parts of the body (metastasis).
Deregulating cellular energetics	They follow the standard pathways to generate energies.	They can make use of abnormal metabolic pathways to generate energy.
Avoiding immune destruction	Immune cells are free to work on them as normal.	They can evade the body's immune system by hiding from it or using "checkpoints" to prevent T cells from attacking tumors.

As already discussed in the table, traits of hallmark of cancer cell, the Figure below is the pictorial representation of the hallmarks of cancer (*Figure 1.1*).

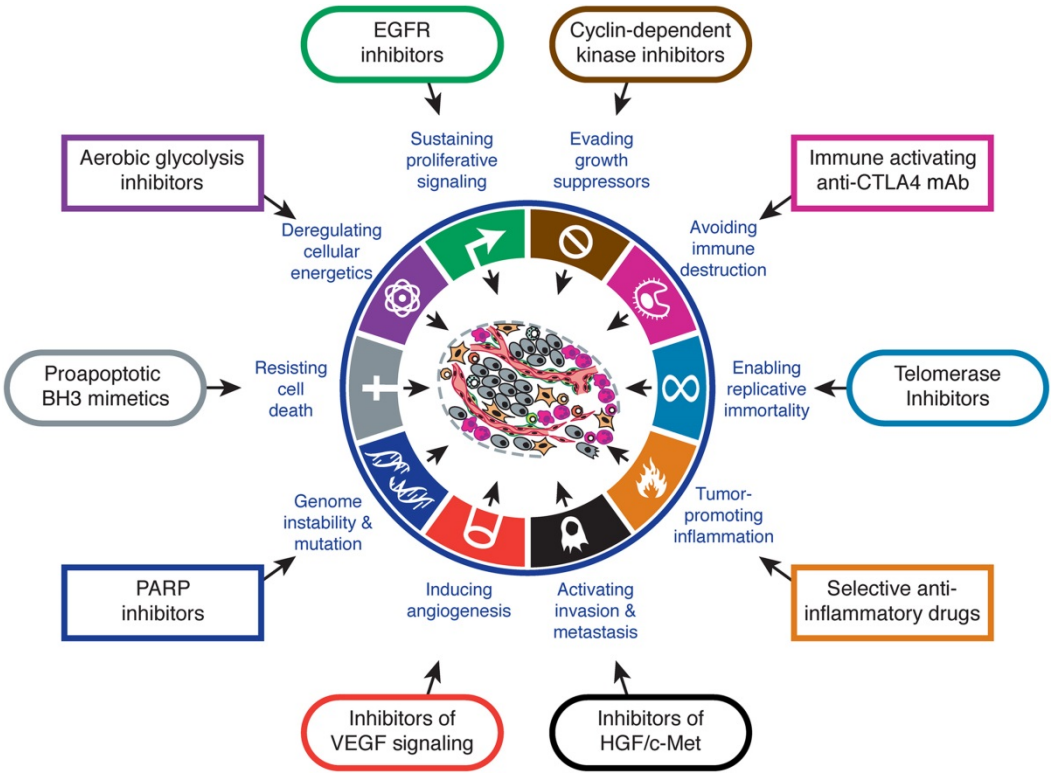


Figure1.1: Therapeutic targets of the hallmarks of Cancer

The Figure is adapted from reference 4

1.3 Causes of cancer:

Cancers are driven from various tissues with different etiologies. Both genetic and epigenetic alterations are equally responsible for the development of cancer phenotypes (*Figure1.2*) [5].

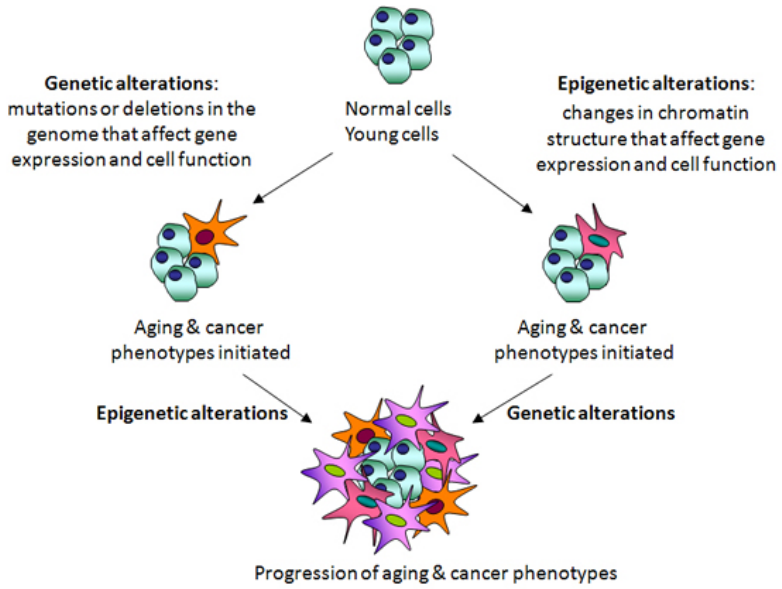


Figure1.2: Genetic and epigenetic alterations for cancer development (Figure adapted from reference5)

1.3.1 Genetic Alterations:

Genetics conceptually deals with gene and gene functions. In general, it is now widely accepted that genetic alterations play a major role in cancer. But the mechanism through which these mutations occur is still being debated. Genetic information is transferred from one cell to another in deoxyribonucleic acid (DNA). DNA from one cell passes the information to mRNA which further leads to protein synthesis [6].

1.3.2 Epigenetic Alterations:

Recent research has revealed that more than 50% of human cancers occur due to mutations in the enzymes that are involved in chromatin organization. Stable heritable changes in phenotype or gene expression in an organism or cell resulting from changes in a chromosome that are not caused by a change in DNA sequence is considered as epigenetic alterations. The process of eukaryotic cell differentiation is one of the most well known examples of epigenetic changes. Tumor cells are not only activated by genetic and epigenetic alterations, but also routinely use epigenetic processes to ensure their escape from chemotherapy and host immune surveillance.

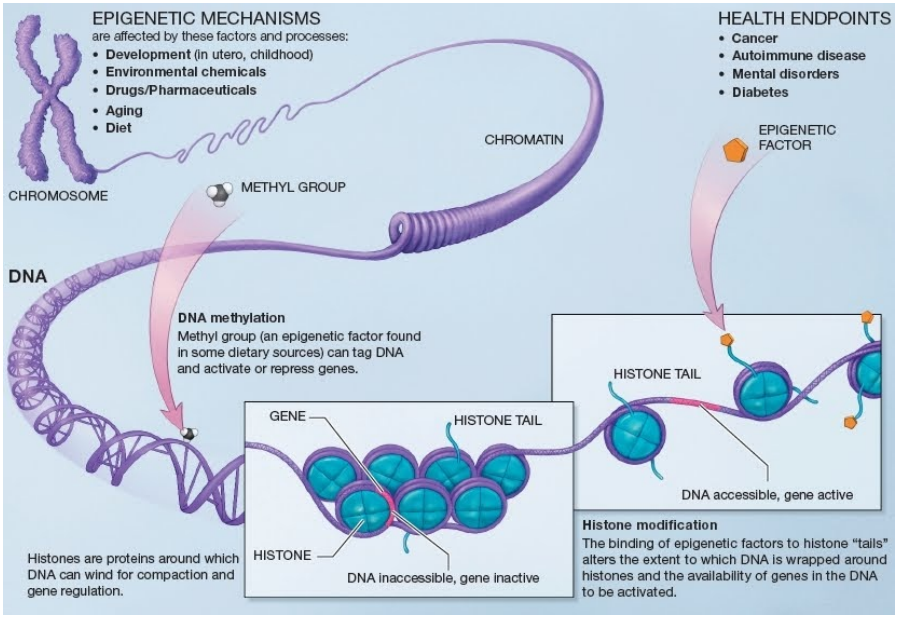


Figure1.3Epigenetic mechanism of Histone methylation; a process for cancer (adapted from Reference 7)

Therefore recently there has been an emphasis by scientists focusing on targeted chemotherapy so as to have minimum side effects. This is leading them to target on epigenome, including DNA methylation and histone modifications. Several drugs are being tested and some have already got approved by the US Food and Drug Administration (FDA) [8]. We should see the increasing success of combining epigenetic drugs with other therapies leading to lesser side effects in future [9]. Epigenetic enzymes catalyze group transfer reactions. Below listed are the enzymes participating in many epigenetic transformations (*Figure 1.4*):

1. DNA methyl transferases (DNMTs)
2. Protein Methyl transferases (PMTs or HMTs)
3. Protein demethylases
4. Histone acetyl transferases (HAT)
5. Histone deacetylases (HDACs)
6. Ubiquitin ligases

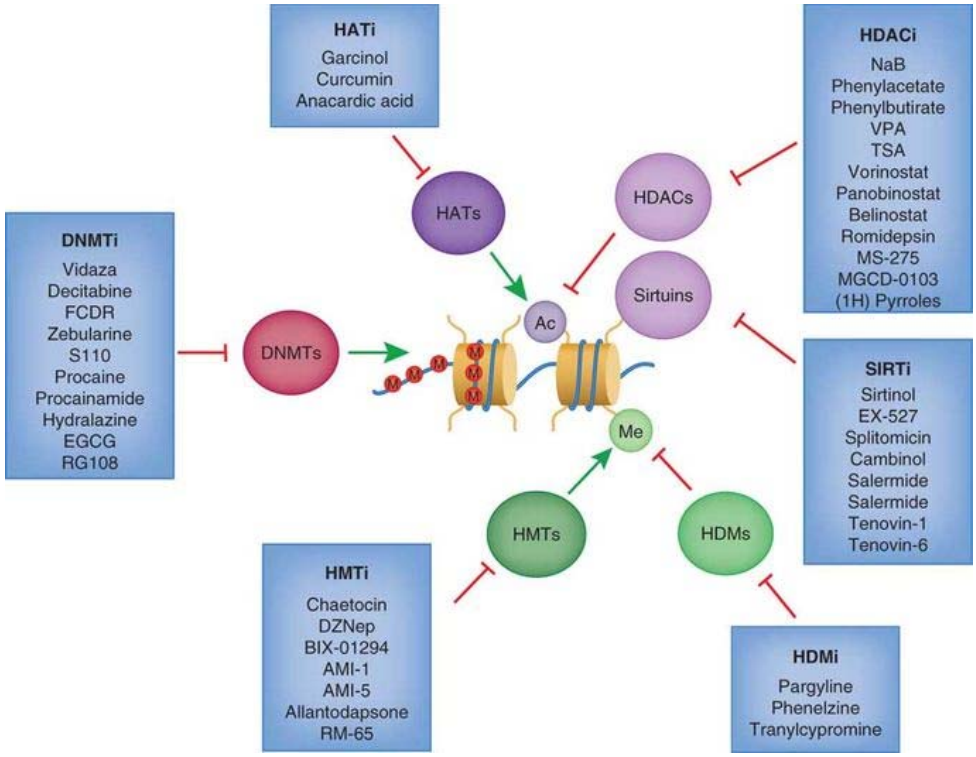


Figure1.4: Enzymes involved in the epigenetic with the drugs targeting it. Structure depicts core histones on which DNA are coiled and the various epigenetic enzymes performing various functions which helps in maintaining cellular activities. Further drugs targeting each enzyme are shown in blue boxes. (Adapted from reference 10)

1.4 Treatment of Cancer

1.4.1 Surgery

Surgery was once considered to be the only option for the cure or to increase the life expectancy for cancer. It involves the removal of cancerous tissues. If the cancer has been diagnosed in early stages the surgery is always the choice of doctors and the most effective treatment. However, the problem is the metastasis occurring due to the leftover cancerous cell after surgery. Nowadays, tissue biopsies are essential for diagnosis and surgery is carried out in 90% of cancer patients [11].

1.4.2 Radiation Therapy

Radiation therapy disrupts atoms within tumour cells by damaging the cell DNA beyond repair. However, the radiations can't distinguish between healthy and cancer cells- especially rapidly dividing cells such as bone marrow and hair follicles so patients suffer from side effects. The advances in research and technology are, however, focusing on minimizing the side effects by precisely targeting the cancer cells [11].

1.4.3 Chemotherapy

In the early 1900s, the famous German chemist Paul Ehrlich worked on developing drugs for infectious diseases. He coined the term "chemotherapy" and defined it as the use of chemicals to treat the disease. Ehrlich wanted to work on developing the drugs for cancer but apparently was not optimistic about the chance for success [12].

Chemotherapy is a treatment used to kill cancer cells. It involves taking one or more of a type of drug that interferes with the DNA of fast-growing cells. These drugs are subdivided into specific classes such as alkylating agents, antimetabolites, anthracyclines and topoisomerase inhibitors.

Current oncological treatments have brought a strong reduction in mortality in cancer patients. However, chemotherapy related cardiovascular complications are well known. Although, all anticancer agents and radiation therapy target tumor cells but they also tend to cause collateral damage to other tissues including the cardiovascular system [13].

A selected snapshot of history is shown in the *Figure1.2*. The major limitations of the drug discovery were: 1. the development of models that can be used to screen the compounds, 2. access to clinical facilities to test such agents. Charles Huggins in 1939 was the first scientist to introduce hormonal therapy based on the observation on the effect of estrogens on breast cancer made by Beatson in 1896. He treated a man with prostate cancer. This exciting piece of work was an important addition to the systematic treatment of cancer and earned Huggins a Nobel Prize.

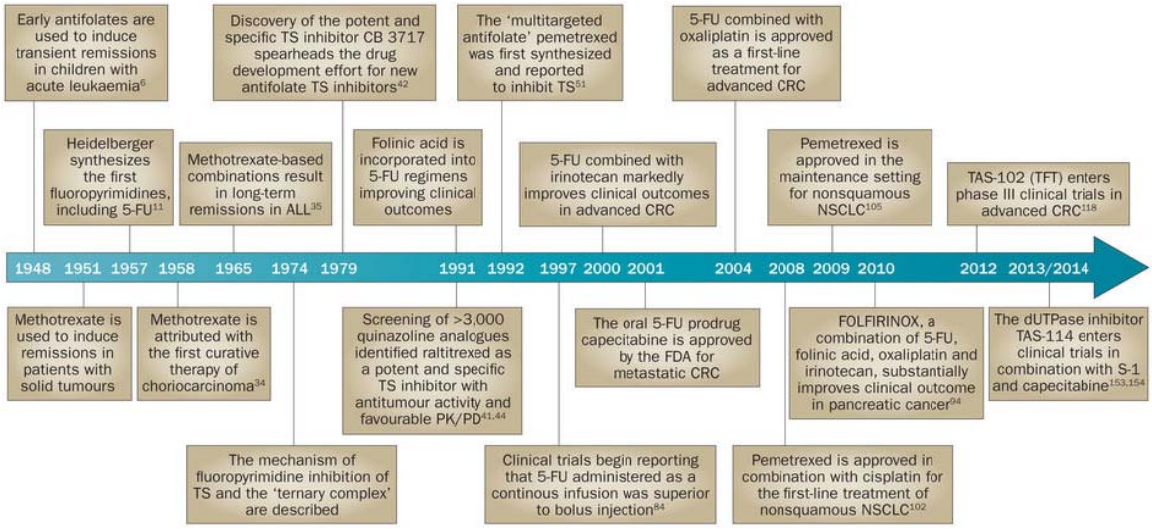


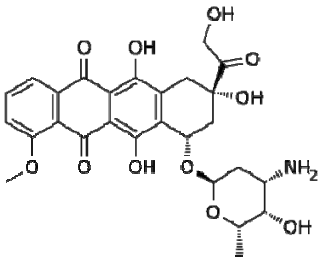
Figure1.5: History of Anticancer drugs

Figure had been adapted from reference14

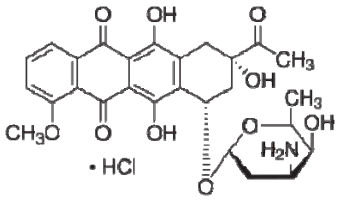
Cancer drugs can be categorized based on the targets of their action as given below:

1.4.3.I DNA interactive compounds

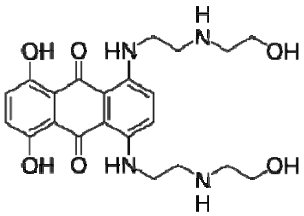
Over the past few decades, the search for new cytotoxic intercalators has mainly followed classical approaches: structure modifications to conventional molecules, new natural products and modifications of them, potential synthetic compounds, and chemical conjugates. Modifications were made to the core structure of traditional intercalators such as doxorubicin, daunomycin and mitoxantrone. (*Figure1.3*)[15].



Doxorubicin



Daunomycin

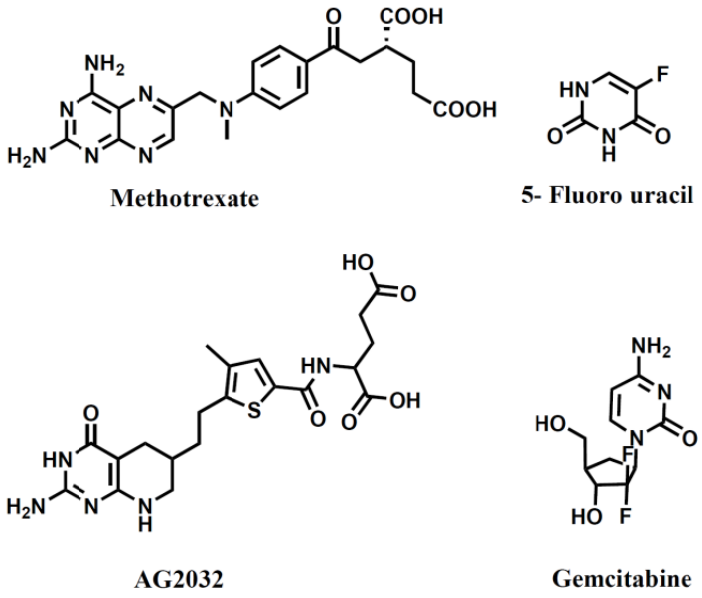


Mitoxantrone

Figure1.6: FDA approved DNA intercalators

1.4.3.II Antimetabolites

Antimetabolites have been in use for the treatment of malignant disease for several years, since the discovery of Farber that aminopterin could cause remission of leukaemia[16,17].An antimetabolite is defined as a drug that interferes with the normal metabolic processes within cells. For example, methotrexate (*Figure 1.4*) is used in the treatment of psoriasis and rheumatoid arthritis. 5-fluorouracil and methothrexate are used as a main stream treatment for solid tumours since several years.



Methotrexate

5- Fluoro uracil

AG2032

Gemcitabine

Figure1.7: FDA approved antimetabolites in market

1.4.3.III Antitubulin agents

Microtubules have several key roles that are important in the process of cell proliferation in eukaryotic cells. For this reason several antitubulin agents have been developed with different purposes, including for use as pesticides, antiparasitics and anticancer agents (*Figure 1.5*) [18].

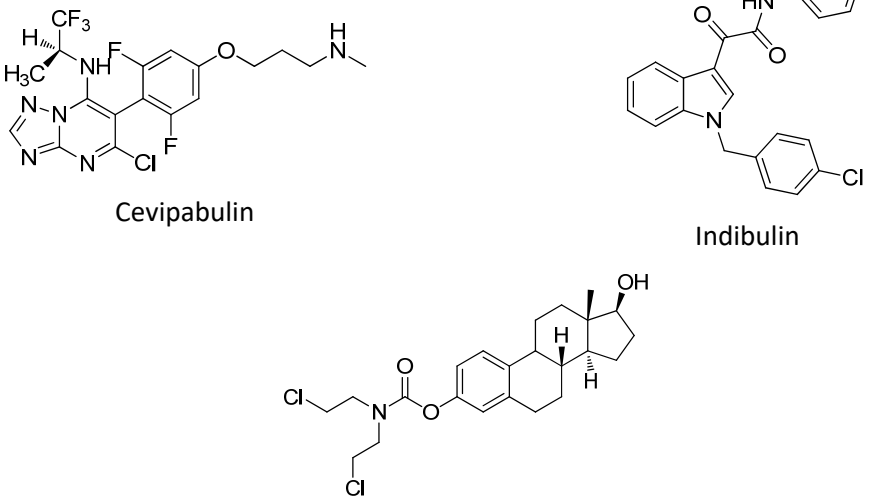


Figure1.8: FDA approved antitubulin agents in market

1.4.3.IVAntihormonal agents

Tumors derived from sexually differentiated tissues such as the endometrium, prostrate and breast can be strongly influenced by the levels of related hormones in the body [2]. Chemotherapy further exploits this influence resulting into the development of various hormonal drugs. As a result Tamoxifen and leuprolide(*Figure 1.6*) are two successful anticancer drugs.

Tamoxifen is known to exert its activity by binding to the oestrogen receptors of the tumour cells. Leuprolide is used in the treatment of prostate cancer.

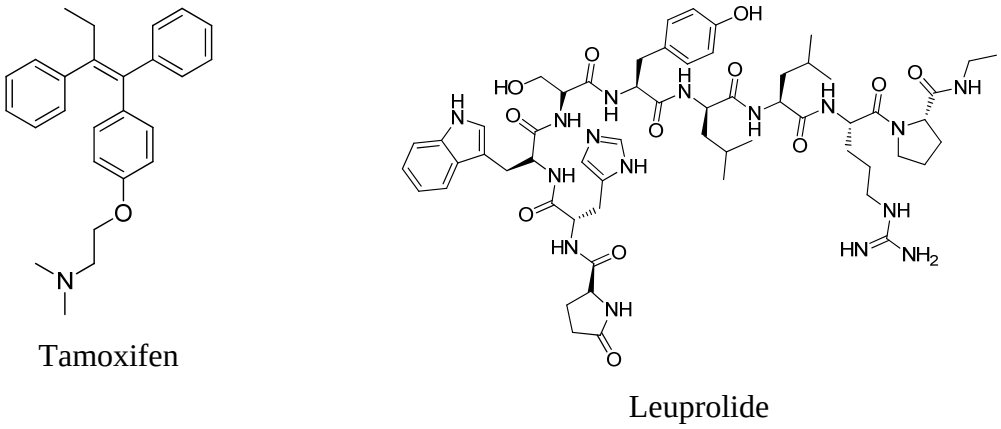


Figure1.9: FDA approved antihormonal agent in market

1.5 Search for new anti-cancer drugs

Scientist will never stop trying from develop a new drug. Working with drug discovery is always challenging, the major reason being the large number of steps involved in this process. Difficulty starts with choosing what to make till the drug is available in the market. The preceding section has given emphasis on various anticancer drugs. As a researcher, there should be a focus on intermediate steps. In this thesis, there is a focus on synthesis of compounds based on the pharmacophore and then screening of the compounds was done.

Steps involved in the final marketing of drug: The layout (*Figure 1.7*) from bench to bedside.

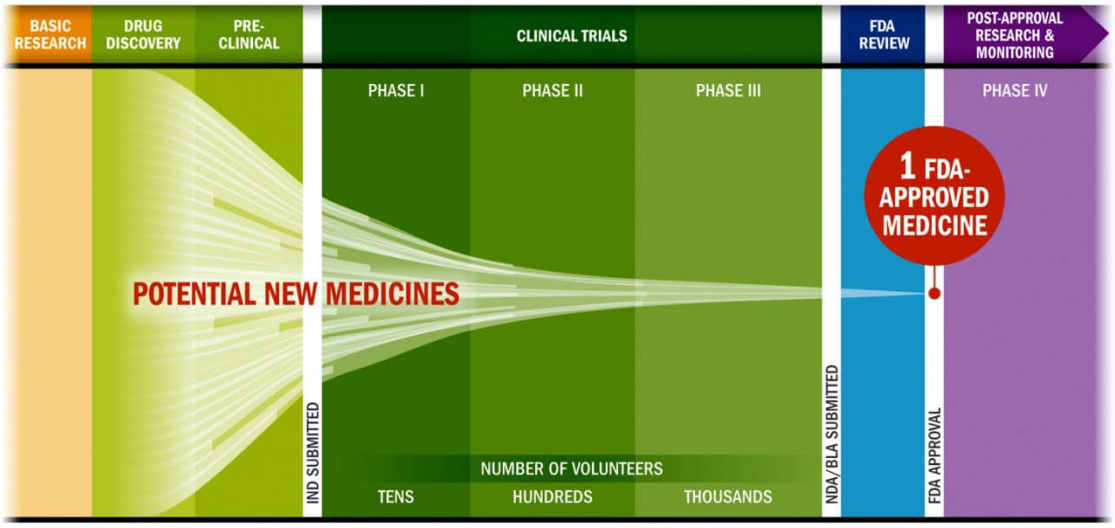


Figure1.7: Layout of final marketed drug (Figure is adapted from reference 19)

IND: Investigational New Drug Application, NDA: New Drug Application, BLA: Biologics License Application

1.6 PAINS:

During the designing of bioactive moieties, Pan-Assay Interference Compounds (PAINS) has been kept in mind PAINS (*Figure1.8*) are getting attention due to ability of certain functional groups causing direct or indirect surge in activity across a range of platforms and against a range of proteins by not discriminating between target and non-target moieties. The most common causes of PAINS activity realized are due to metal chelation,

chemical aggregation, redox activity, compound fluorescence, or promiscuous binding[20,21] .

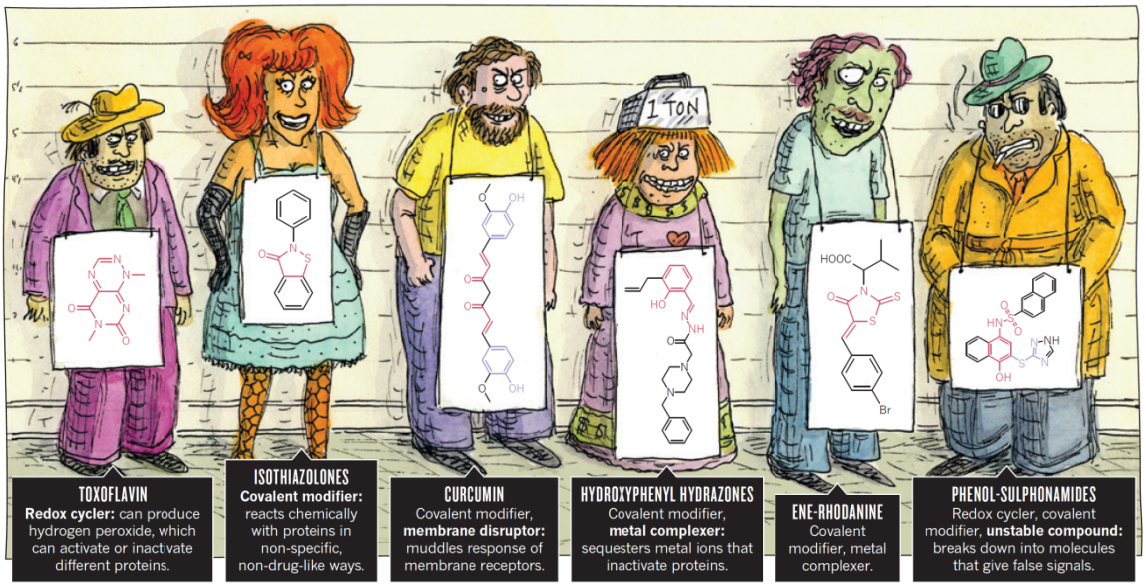


Figure1.8: In red showing the pains functionality

Figure adapted from reference [16]

1.7 Rational of the work/ Research Objectives

The focus of this work is to design and synthesize novel challenging molecules for anti-cancer. The specific aims of this thesis are:

- To design novel molecule taking into consideration PAINS structures with one or more than one pharmacophores. (Ideally to understand synergy between pharmacophores.)
- Develop schemes for designing target molecules using retrosynthetic strategy, so as to achieve the maximum yield with environment friendly method.
- To investigate the influence of solvent on the intermediate steps so as to achieve the maximum yield.
- To model interactions of the designed molecules with the target enzyme (PRMT1; Protein Arginine Methyl Transferases 1).

- To check the anti-proliferative activity of the synthesized compounds on human cancer cell lines. Correlate the activity with the structure and get a more clear insight for the designing.
- Understanding structure (structure-activity relationship) for one of the marketed anti cancer drug Letrozole, by employing novel crystallization methods.

1.8 Thesis outline

The structure of this thesis is as follows:

Chapter 2 contains five sub chapters 2.1, 2.2, 2.3, 2.4, 2.4 and 2.5. This chapter focuses on designing, synthesis, characterization and biological activity of novel compounds with different pharmacophores.

Chapter 2.1 describes the relevant literature in brief for the synthesis of un-substituted amidines. Here we present a method for conversion of nitrile to amidine in single step and in the presence of amide functionality using metal amide and/or ammonia gas. This method is extended for the synthesis of ten new amidines-amide conjugates where strategy works effectively in presence of heterocyclic functionality as well. Apart from nearing room temperature, most of the time yield observed in the modified reaction conditions clocks above 70% for nitrile to amidine conversion. Our preliminary results confirmed thatamidine-amide conjugates can act as anti-proliferative active compounds. All the compounds were well characterized using CHN, FT-IR, NMR and Mass analysis. Docking studies performed on PRMT1 showed one of the derivatives gets U type bend inside the cavity for extra π-cation interaction. Single crystal for one of the compound was developed and solved. The MTT assay was performed on HeLa cell line to check there anti-proliferative activity. Also these compounds were screened on 60 cancer cell lines for broader aspect. Our results, manifest that heterocyclic derivatives bridged by –methylene groups, similar to five membered ring containing furfuryl derivative and six membered ring containing picolyl derivative**,** to amidine-amide system should be worth investigating as potential anticancer agents.

Chapter 2.2 presents click reaction to conjoin triazole pharmacophore with benzimidazolein a faster and efficient way. The reaction proceeds without isolation of azide intermediate, *in-situ* click chemistry, without compromising in good yields. Synthesis of eleven new compounds bearing triazole and benzimidazole derivatives of *N*-((1-((1*H*-benzo[*d*]imidazol-2-yl)methyl)-1*H*-1,2,3-triazol-4-yl)methyl)aniline is achieved. The *in-vitro* anticancer activity of selected compounds using NCI-60 human cell line screening program reveals that most of the title compounds show moderate bioactivity at 10μM concentration. Flexible structured molecule *N*-((1-((1*H*-benzo[*d*]imidazol-2-yl)methyl)-1*H*-1,2,3-triazol-4-yl)methyl)-4-chloroaniline show a 40% growth inhibition in human renal cancer cell line (UO-31) which needs further investigation. Crystal for one of the derivatives is developed and solved. Conformational polymorphism for one of the derivatives is observed and is under detailed investigation. Crystal structure revealed interesting hydrogen bonding between NH-O and OH-N.

Chapter 2.3 presents the synthesis of four pyrimidine bearing compounds linked aniline derivatives. Pyrimidine drugs are used for the treatment of three main disease classes: anti-infective, cardiovascular, and oncological. The designing of compounds, is driven by three different ways: (i) 2,4-linked pyrimidine derivatives with dimethyl amine group fixed at position 2; (ii) 4,6-linked pyrimidine derivatives with dimethyl amine fixed at position 4 and (iii) use of derivatives of aniline in both above cases. Overall synthesis is carried out in two steps. In first step 2,4- or 4,6- dichloropyrimidine is treated with aniline and 4-methoxy aniline under ambient conditions. Second step being S_NAr reaction is performed to insert dimethylamine group either by direct insertion or by *in situ* formation. Single crystal of one of the derivative has shown planar structure.

Chapter 2.4 presents the synthesis of three novel benzimidazole linked aniline derivatives. Cyclisation of respective diamine group followed by two substitution reactions to give the required product molecules is the overall strategy. Construction of N-((1*H*-benzo[*d*]imidazol-2-yl)methyl)-4-methyl-*N*-(4-methylbenzyl)aniline and its derivatives were achieved in high yields.

Chapter 2.5 presents the synthesis of amidine linked quinoxalinone derivative, initially planned for amidine but amidine functionality was not obtained but we landed up in the synthesis of novel cyano derivative, 4-methyl-3-oxo-2- (p-tolyl)-3,4-dihydroquinoxaline-6-carbonitrile. Cyano-*o*-phenylenediamine with glyoxalic acid furnished quinoxalinone in 95% yield. Next step is C-H bond activated Suzuki coupling reaction with boronic acid derivatives finally furnished the desired compound, 4-methyl-3-oxo-2- (p-tolyl)-3,4-dihydroquinoxaline-6-carbonitrile. Hence the overall synthesis of cyano-quinoxalinone was achieved in eight multiple steps with good yields.

Chapter 3 reports the self aggregation behavior of benzimidazole linked triazole adducts in NMR tube. Systematic study were undertaken to unravel this behavior on eight designer derivatives. The synthesis of these molecules is carried out by modulating three facts: (i). flexibility effect of phenyl/aniline linkers; (ii). the effect of introducing 'fluoro' functionality; and (iii). addition of ethyl group on benzimidazole nitrogen. All these molecules showed long fiber like structure, which makes it semi-solid or gel like, with change in concentration in DMSO solvent and/or addition of water. Fibers obtained from DMSO are much thicker as compared to water addition fibers. A microscopic study concluded that the growth of fibers is sperulitical initially and then fibrillar with both tip branching and side branching. Thermal analyses TG-DTA and DSC showed phase transitions at above 100°C, for the three representative molecules. Single crystal X-ray study revealed absence of non-covalent interactions for two of the ethyl derivatives in solid phase. COSY and HSQC NMR measurements are used for the complete assignment of proton signals for all newly synthesized eight benzimidazole linked triazole molecules in DMSO. Concentration dependent and water addition proton NMR experiments revealed the specific shifting of peaks, such as methylene bridges and *ortho*-protons of the phenyl ring. Thus, these protons may be the probable candidates for aggregation or self-aggregation behavior. Close-contact in Single crystal XRD studies and thermal data supports our NMR conclusions. The fibers are stable at room temperature for more than a week's time and their formation is thermoreversible.

Chapter 4 reports crystallization of Letrozole, a well marketed drug for hormone receptor positive breast cancer. Letrozole molecule in-spite of having many pro-

hydrogen-bonding sites and planar aromatic ring did not show their 'expression' in actual solid state crystal structure. Systematic proportions of hydroquinone and pyrogallol were planned for the same during slow crystal growth experiment. Detailed investigation of thus obtained products using DSC is performed, which showed interesting small but distinct shifting of endotherms. This result is well supported by single crystal data and powder XRD pattern of already reported forms reveals observation of new polymorph. New polymorph crystallizes in orthorhombic crystal system with *C-1* space group. New polymorph can differ in solubility behavior, thermal stability, chemical inertness and hence bioavailability of the marked drug.

Annexure I presents the synthetic strategy for novel triazole and *o*-hydoxy aniline adducts derivatives of *N*-(2-hydroxyphenyl)-2-(4-phenyl-1*H*-1,2,3-triazol-1-yl)acetamide. Three new molecules are synthesized. Synthesis starts with condensation of chloroacetylchloride with *o*-hydroxy aniline. The next step is the azide formation followed by Click reaction to give the desired product. Overall yields of 30-40% were achieved. Single crystal of one of the intermediate compound was developed and solved.

Annexure II presents efforts we put in protein isolation work which is performed in collaboration. Knowing the protein-drug interaction gives good insights in designing the best possible drugs with minimum side effects. Having this in mind we have developed a clone to give us the required protein PRMT1.

In summary, the thesis presents the overall synthesis of novel molecules with six different pharmacophores, normally observed in anti-cancer drugs: A. Amidine, B. Benzimidazole and triazole adducts, C. Pyrimidine, D. Benzimidazole, E. Quinoxazole F. Triazole and aniline adducts. Designing of the novel molecules is done taking PAINS structures into consideration. The synthesis is designed to obtain over all good yields. Characterization of all the newly synthesized molecules were carried out using CHN, FT-IR, ^{1}H and ^{13}C NMR, ESI-MS/HRMS and where ever necessary by single crystal XRD analysis. The docking studies have been performed for amidine derivatives. Synthesized molecules have been tested for their anti-proliferative activity using MTT assay and NCI-60 human cancer cell line screen. The observed IC_{50} values for the molecules shows the validity of

the concept in designing and synthesis of novel molecules based on pharmacophore drug designing. An attempt to synthesize target protein PRMT1: Protein Arginine Methyl Transferases was made in collaboration. This thesis also uncovers (1) detailed investigation (using 2D NMR, DSC, microscopic imaging) of serendipitous assembly formation of triazole and benzimidazole adduct in presence of water; and (2) serendipitous discovery of polymorphism in Letrozole, a standard anti-cancer drug.

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