Chapter 1

Introduction
1.1 Statistics and definition of Cancer

World Health Organization’s report of Global and regional mortality due to diseases indicates 56.4 million deaths in 2015, of which deaths due to ischemic heart disease is the most leading cause of deaths with 15 million deaths and cancer is second leading cause of disease linked deaths with 14 million deaths. The number of new cancer cases is suspected to increase by 70% in the next 2 decades. In next few years death due to cancer will cross that of deaths due to heart disease and will become the leading cause of death (National Center for Health Statistics-2015).

Cancer is a disease involving abnormal cell growth forming malignant tumor with the potential to invade nearby tissues, lymph nodes and other organs. Cancer cells migrate to distant parts of the body through blood vascular system and start a secondary tumor at that location, a process termed metastasis ("Cancer Fact sheet N°297". WHO,"Defining Cancer". National Cancer Institute). A neoplasm or tumor is a group of cells that have undergone abnormal and uncontrolled growth which in turn leads to form a mass or a lump. These can also be found diffused sparsely ("Cancer Glossary". Cancer.org. American Cancer Society, Cancer.gov. National Cancer Institute). Tumor cells have six hallmark characteristics which are required to transform them into malignant tumor. They include sustaining proliferative signaling, evading growth suppressors, evading apoptosis, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan D. et al., 2000, Hanahan D. et al., 2011). The sign and possible symptoms of the cancer include a lump, abnormal bleeding, prolonged cough, unexplained weight loss and a change in bowel movements (Cancer-Signs and symptoms- NHS Choices). The majority of cancers, 90–95% of cases, are due to environmental factors and remaining 5–10% is due to inherited genetic changes. Common environmental factors that contribute to cancer deaths include tobacco (25–30%), diet and obesity (30–35%), infections (15–20%), radiation (both ionizing and non-ionizing, up to 10%), stress, lack of physical activity and environmental pollutants (Anand P. et al. 2008). Exposure to particular substances has been linked to specific types of cancer. These substances are called carcinogens.
1.2 Carcinogens

Tobacco smoke contains over 50 known carcinogens which cause 90% of the lung cancer and other various types of cancer (Biesalski HK. et al., 1998, Kuper H. et al., 2002). Diet, physical inactivity and obesity are responsible for 30–35% of cancer affected deaths (Anand P. et al., 2008, Kushi LH. et al., 2006). Approximately 18% of cancer deaths are related to infectious diseases (Anand P. et al., 2008). Viruses are the usual infectious agents that cause cancer but cancer bacteria and parasites may also play a significant role. Viruses that can cause cancer called oncovirus which include human papilloma virus (cervical cancer), Epstein-Barr virus (naso-pharyngeal carcinoma), Kaposi’s sarcoma virus (Kaposi’s sarcoma) and hepatitis B and hepatitis C viruses (hepatocellular carcinoma). Literature reports confirm the introduction of Helicobacter pylori induced gastric carcinoma (Pagano JS. et al., 2004, Ljubojevicet et al., 2014). Up to 10% of invasive types of cancer are related to exposure to radiation, including both ionizing and non-ionizing radiations (Anand P. et al., 2008). Most of the cancers are non-hereditary (sporadic), only 3-10% of the cancers are hereditary which are primarily caused by an inherited genetic defect. Mutations in BRCA1 and BRCA2 genes account for more than 75% risk of breast, ovarian and hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome) (Roukos DH., 2009, Cunningham D. et al., 2010). Some substances cause cancer primarily through their physical, rather than chemical effects. Asbestos, naturally occurring mineral fibers, non-fibrous materials like powdered metallic cobalt and nickel and crystalline silica (quartz, cristobalite and tridymite) may cause cancer (Maltoni CF. and Holland JF. Chapter 16: Physical Carcinogens-2000). Frequent, long-term application of hot objects to the body, frequent consumption of scalding hot tea may produce esophageal cancer. Some hormones play a role in the development of cancer by promoting cell proliferation. Insulin-like growth factors and their binding proteins play a key role in cancer cell proliferation, differentiation and apoptosis, suggesting possible involvement in carcinogenesis (Henderson BE. et al., 2000 - Hormones and the Etiology of Cancer, Rowlands et al., 2009).
1.3. Cancers are classified based on their tissue of origin

Carcinoma derived from epithelial cells, sarcoma derived from connective tissue (Bone, cartilage, fat and nerve), Lymphoma and leukemia derived from hematopoietic cells that leave the marrow and tend to mature in the lymph nodes and blood, respectively, Germ cell tumor derived from pluripotent cells present in the testicle or ovary (Seminoma and dysgerminoma respectively) and Blastoma derived from immature "precursor" cells or embryonic tissue.

1.4 Cancer Initiation and Progression

Cancer develops in three major steps initiation, promotion and progression. Cancer initiates with multiple successive mutations due to exposure with initiator carcinogens (UV-light, ionization radiation, thermal disruption, or chemical sources) and its accumulation. If DNA remains unrepaired, further exposure with promoting agents causes clonal-expansion. Once a tumor establishes then further mutations, genetic instability and epigenetic changes results in expansion of cancer (Hanahan D. et al., 2000, Hanahan D. et al., 2011).

1.5 Cancer associated Genes

There are three major groups of genes in which either genetic or epigenetic changes drive a normal cell into a malignant cell: Tumor suppressor genes, proto-oncogenes and DNA repair genes (Ciardiello F. and Tortora G., 2008).

i. Tumor suppressor genes are a group of genes that prevent a normal cell to change into a malignant cell. Loss of function of tumor suppressor genes is reported in various human cancers (Weinberg RA., 2014). Tumor-suppressor genes (Sherr CJ., 2004) play various roles in cell cycle regulation, coupling the cell cycle with DNA damage repair, if the damage irreparable or promote the apoptosis. Another role of tumor suppressor genes is to regulate metastasis. Proteins involved in cell adhesion usually regulate metastasis, prevent tumor cells from dispersing, block loss of contact inhibition, and finally inhibit metastasis (Yoshida BA. et al. 2000, Hirohashi S. et al., 2003). Retinoblastoma (RB), TP53, Phosphatase and tensin homolog (PTEN), HNPCC, MEN1, BRCA and SWI/SNF important tumor suppressor genes (Markowitz S.,
Tumor suppressor genes can also be regulated by post-transcriptional regulation mediated by micro-RNAs (miRNAs) (Negrini M. et al., 2007).

ii. Proto-oncogene: A class of genes that were active in certain developmental stages and remain inactive there after. In case of cancer proto-oncogene are activated and induces cell growth, differentiation and often involved in signal transduction and cell cycle regulation. Due to mutation, a proto-oncogene can change into oncogene (Todd R. et al., 1999). RAS, WNT, MYC, ERK, and TRK are major proto-oncogenes, mutation or epigenetic changes in this proto-oncogenes lead to various human cancers. Expression of oncogenes can be regulated by micro-RNAs (miRNAs) (Negrini M. et al., 2007).

iii. DNA repair genes: A large group of genes are associated with identification and correction of damaged DNA or Chromosome. All these genes are considered as the DNA Repair genes and the process collectively is known as DNA repair (Lodish H. et al., 2004). When DNA damaged beyond repair and cellular apoptosis does not occur than cells may change into malignant (Acharya PV., 1971, Bjorksten J. et al., 1971). DNA-repair genes are frequently mutated in cancer. ATM (Ataxia telangiectasia mutated) and ATR (Ataxia and Rad-related) are kinases associated with the regulation of cell cycle checkpoint activation. ATM responds to the DNA double strand breaks and disruptions in chromatin structure where as ATR respond to stalled replication forks (Bakkenist CJ. et. al., 2003). In most of the tumor cells ATM is often absent or down-regulated because of hyper-methylation. BRCA1 and BRCA2 are associated with many of the DNA-repair mechanisms such as non-homologous end joining (NHEJ) and homologous recombination. Mutation in BRCA1 and BRCA2 increases the risk of breast cancer on carriers. Epigenetic defects were found in the BRCA1 gene in the tumor cells (Baldassarre G. et al., 2003, Li D. et al., 2014). RAD51 and BRCA2 are associated with the homologous recombination repair process and both of the gene expression was down-regulated in various types of cancer (Tutt AN. et al., 2003). ERCC1, associated with nucleotide excision repair process is epigenetically suppressed and down-regulated in colorectal cancer (Dolle et al., 2006). MSH 2, 3, 4 are components of the DNA mismatch repair system and are
epigenetically down-regulated in various types of cancers (Hegan DC. et al., 2006, Lee KH. et al., 2011). In case of colorectal cancer, loss of PMS2 expression is likely due to epigenetic over-expression of the miRNA, miR-155, which down-regulates MLH1 (Negrini M. et al., 2007, Esquela-Kerscher A. and Slack FJ., 2006).

1.6 Colon and rectum

Colon and rectum are part of digestive system. The collective part begins from the end of small intestine and ends to anal opening. It is also called large intestine (Large bowel). As shown in figure 1.1. A colon begins from Cecum, Vermiform diverticulum also called Appendix is located in the lower caecum then ascending colon, transverse colon, descending colon and ends with sigmoidal part of the colon (Large Intestine NCI-Dictionary- NIH, Kapoor and Vinay k., 2011, Gray Henry, Gray's Anatomy, 1918). The function of colon is to absorb water and store the waste product of digestion until it is ready to defecate (The Large Intestine (Human) News-Medical.net. 2009). Upper rectum part is called sacrum which is similar in diameter like the sigmoidal colon but lower part of rectum is wider and extends to a sac like structure called ampulla. The key role of ampulla is to store fecal matter (Bruce G. W. et al., 2007). Due to storage of faces in rectal ampulla the wall of rectal ampulla get expanded which causes a stretch receptor of the rectal wall stimulation. This leads to sensation of defecation (new health guide. org-Rectum-Function). The anatomy of colon and rectum are almost similar, made up of different layers. As shown in figure 1.1. B lumen surface is lined by mucosa (a thin layer of epithelial cell). Below mucosa a layer of connective tissue called lamina propria and a thin layer of muscle called muscularis mucosa. Submucosa is made up of a connective tissue which surrounds mucosa and contains mucus glands, blood vessels, lymph vessels and nerves. Muscularis propria lies outside of submucosa which is made up of a thick layer of muscle and it has an inner ring of circular muscle fibers and an outer ring of long muscle fibers. Serosa is the outermost layer of colon but it is not found on most of the rectum part (Edge SB. et al., 2010).
1.7 Colorectal cancer statistics

GLOBOCAN 2012 estimated 14.1 million new cancer cases and 8.2 million cancer related deaths which occurred in 2012. This data can be drawn in comparison with 12.7 million and 7.6 million, respectively, in 2008. Worldwide the most commonly diagnosed types of cancer were those of the lung (1.8 million, 13.0% of the total), breast (1.7 million, 11.9%), stomach and colorectal (1.4 million, 9.7%). Globally more than 1 million people get colorectal cancer every year resulting in about 715,000 deaths as of 2010 (Cunningham D. et al., 2010). This number has risen from 490,000 as reported in 1990 (Lozano R. et al., 2012). As of 2012, it is the second most
common cause of cancer in women (9.2% of diagnoses) and the third most common in men (10.0%) (World Cancer Report, 2014, International Agency for Research on Cancer, World Health Organization, 2014). Overall it is the fourth most common cause of cancer death after lung, stomach, and liver cancer (WHO -2010 "Cancer"). It is more common in developed countries than that of the developing countries (Merika E. et al., 2010). Globally, the highest incidence was reported in Australia, New Zealand, Europe and the US. The lowest rates were reported in Africa and South-Central Asia (Colorectal Cancer Incidence, Mortality and Prevalence World wide in 2008). Along with Lung, stomach and liver, colorectal cancer gradually becomes a serious and challenging problem and it desperately needs attention for the improvement in the treatment strategy.

1.8 Basics of colorectal cancer

Colorectal cancer (CRC) also known as bowel cancer, is the cancer developed from the colon or rectum. Signs and symptoms may include blood in the stool, a change in bowel movements, weight loss, and feeling of continuous fatigue. Most of the colorectal cancer is due to old age and lifestyle factors with only a small number of cases coming due to underlying genetic disorders. Some risk factors include diet, obesity, smoking, and lack of physical activity (World Cancer Report (WHO), 2014). Dietary factors that increase the risk include red and processed meat and alcohol. Another risk factor is inflammatory bowel disease, which includes Crohn's disease and ulcerative colitis. Some of the inherited genetic disorders that can cause colorectal cancer include familial adenomatous polyposis and hereditary non-polyposis colon cancer. However, these comprise less than 5% of cases. It typically starts as a benign tumor, often in the form of a polyp, which over time becomes cancerous (World Cancer Report (WHO), 2014).

1.9 Stage classification of colorectal cancer

Colorectal cancer initiates on the luminal (mucosa) surface of colon and rectum and spreads to sub mucosal, muscular layer, nearby tissue organ, local lymph nodes and finally to distant parts through vasculature. Progression of CRC is classified, based on TNM staging from stage 0 to 4. Tumor initiation and progression start on the
mucosal surface (Stage 0) that invades to sub mucosal layer in stage-I. After that it reaches the muscular layer and spreads to nearby tissues and organs in stage-II. Further progression of tumor leads to migration of cancerous cells to lymph nodes in stage-III and finally spreads to distant part of the body (Metastasis) in stage-IV) (Centelles JJ., 2012, Edge SB. et al., 2010).

1.10 Colorectal cancer management

There are different therapeutic strategies are available for the management of colorectal cancer. Common treatments include surgery, radiation therapy, chemotherapy, targeted therapy and different combinations of the above method. The treatment advised usually depends on the stage of the cancer. Cancer that is confined within the wall of the colon can be cured by surgery while cancer that has spread widely is usually not curable by surgery alone. For stage I colon cancer, chemotherapy is not advised, and surgery is the definitive treatment. Chemotherapy is rarely used in stage II colon cancer and is offered only when risk factors identified. Chemotherapy is the primary mode of treatment for stage III and stage IV colon cancer. In the case of colon cancer, radiotherapy is usually avoided due to sensitivity of the bowels to radiation but a combination of radiation and chemotherapy may be recommended for rectal cancer (Cunningham D. et al., 2010, DeVita et al., 2008 cancer: principles & practice of oncology 8th ed.). Radiotherapy can be used in neo-adjuvant (chemotherapy before surgery) and adjuvant (post surgery chemotherapy) settings for some stages of rectal cancer. Targeted therapy is another treatment modality that includes immunotherapy and small molecule based multi-kinase inhibitors as therapeutics.

i. Surgery of CRC tumor: Primary colon cancers are mainly treated by surgery involving complete meso-colic excision (CME) (Roukos DH., 2009, Cunningham D. et al., 2010) along with arteries and veins similar to total meso-rectal excision (TME) for rectal cancer in stage II, and stage III (François J. et al., 2012). Colonic segmental resection (Right hemi-colectomy, transverse colectomy, left hemi-colectomy or total colectomy) is performed according to the site of the tumor. The surgical procedure depends on the stage of the colorectal cancer. For stage-I colorectal cancer in which tumors invade to mucosa and sub-mucosa layer, only local surgery is recommended
and adjuvant chemotherapy is usually not required (Maltoni C. F. and Hollard J. F. Chapter 16: Physical Carcinogens, 2000). Later stage of Stage-II and earlier stage of stage-III of the CRC require additional complete meso-colic and total meso-rectal excision along with postoperative neo-adjuvant radio-chemotherapy and adjuvant chemotherapy to reduce chances of recurrence (Cunningham D. et al., 2010). Pre and post-operative radio-chemotherapy is recommended in later stage of stage-III and stage-IV of colorectal cancers.

**ii. Radiotherapy:** Radiation is used to kill cancer cells by generating free radicals and depositing energy into cells. X-rays are generated by devices that excite electrons (e.g. cathode ray tubes and linear accelerators), while gamma rays originate from the decay of radioactive substances (e.g. cobalt-60, radium and cesium). Both X-rays and Gamma rays are photon radiations. Particle radiations such as electron beams are used to treat surface tumors because of their low penetrating power. Ionizing radiation either directly damage cellular DNA or indirectly damage the DNA by generating free radicals thereby causing cancer cells to lose their ability to proliferate (Baskar R. et al., 2012, Jackson SP. and Bartek J., 2009) and finally activating processes inducing cell death. Radiation does not specifically target only cancer cells and can also cause damage to normal cells. Since cancer cells divide rapidly and the interphase part of the cell cycle is very short or absent, they fail to repair the damaged part of DNA. In contrast, normal cells divide more slowly and have long interphase providing sufficient time for DNA repair. Additionally, the DNA repair mechanism in cancer cells becomes very weak and they fail to repair the damaged DNA. Therefore, the radiation therapy mostly kills cancer cells along with very few normal cells (Baskar R. et al., 2012, Begg AC. et al., 2011). Ionizing radiations cause single or double strand breaks in the DNA. Single strand breaks can be easily repaired but double stand breaks are very difficult to repair and it is a time taking process. In cancer cells (Fast and uncontrolled proliferating cells), unrepaired double strand breaks lead to genetic instability and activation of death process. Clearly, double strand breaks are the primary cause of cancer cell death during radiation therapy (Baskar R. et al., 2012). Radiation can be focally administered at the tumor location either by high energy external beam radiation delivered from
outside the body or by internal radiation (brachytherapy) delivered by a radioactive source sealed into a catheter or seeded directly into the tumor (Baskar R. et al., 2012). Novel radiation therapy techniques are being developed such as advanced imaging, sophisticated software and optimal dose delivery systems that maximize tumor killing and minimize damage to normal cells. 3D Conformal radiotherapy (3DCRT) is the novel 3D radiation therapy is based on CT imaging and has replaced 2D radiation therapy that used rectangular fields on plain X-ray imaging. The 3D approach locates and estimates the size of the tumor more accurately to enable precise targeting with radiation beam and protecting the neighboring tissue from radiation exposure (Wang-Chesebro A. et al., 2006). Intensity modulated radiation therapy (IMRT) is used to create irregular shaped radiation doses that conform to the tumor and simultaneously avoid exposure to critical organs (Feng FY. et al., 2007). Image-guided radiotherapy (IGRT) uses information acquired through pre-radiotherapy imaging to guide radiotherapy and is used when critical structures, tissues or organs are close to the tumor and even a slight positional error may be unacceptable (Langen KM. et al., 2001). Stereotactic body radiation therapy (SBRT) is based on the above technological advancements, and allows precise delivery of very high individual doses of radiation to target well-defined primary and oligo-metastatic tumors and can be administered anywhere in the body (Jaffray DA. et al., 2002).

iii. Chemotherapy: Chemotherapy is the administration of anticancer chemical drugs either singly or in different combinations. Commonly used anti colorectal cancer chemotherapeutic drugs include 5-Fluorouracil (5-FU), Capecitabine, Leucovorin, Methotrexate, Platinum compound Oxaliplatin and Irinotecan. Chemotherapeutic drugs are used before or after surgery and sometimes both before and after surgical intervention. Pre-operative treatment is preferred as neo-adjuvant chemotherapy in advanced stages of colorectal cancer to shrink the tumor size prior to surgery and is usually administered in combination with radiotherapy as either short course or long course chemo-radiotherapy depending on the patient’s age as well as the grade and location of tumor. Postoperative chemotherapy (Adjuvant) chemotherapy is commonly recommended to minimize the possibility of recurrence by ensuring the death of any cancer cells that remain after surgical resection in the primary tumor location or in the blood stream.
**a. 5-Fluorouracil (5-FU):** 5FU is an anti-metabolite drugs and analogue of Uracil with a fluorine atom at the C-5 position in place of hydrogen (Figure 1.2). 5-FU uses the same facilitated transport mechanism as Uracil to rapidly enter the cell (Daniel BL. et al., 2003, Wohlhueter RM. et al., 1980). Uracil is normally incorporated into RNA and methylated to generate thymidine for DNA replication (Diasio RB. & Harris BE., 1989). After drug administration, during the transcription process, 5-FU gets incorporated into RNA instead of Uracil and due to the presence of F-atom on the 5-C position of Uracil; it fails to convert into Thymidine. Hence, this analogue of Uracil inhibits both transcription and replication processes.

**b. Thymidylate Synthase (TS):** TS is an enzyme that catalyzes the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), with the reduced 5, 10-methylentetrahydrofolate (CH2THF) as the methyl donor. TS is a dimeric protein, both subunits contain a nucleotide-binding site and a binding site for CH2THF. The 5-FU metabolite FdUMP forms a stable ternary complex by binding to the nucleotide binding site of TS and CH2THF, thereby blocking binding of the normal substrate dUMP and inhibiting dTMP synthesis (Sommer H. and Santi DV., 1974). Thus, 5-FU exerts its anticancer effects by inhibiting TS and incorporating
wrong metabolites into RNA and DNA. The rate limiting enzyme in 5-FU catabolism is dihydropyrimidine dehydrogenase (DPD), which converts 5-FU into inactive dihydrofluorouracil (DHFU). More than 80% of administered 5-FU is primarily catabolized in the liver, where DPD is abundantly expressed (Daniel BL. et al, 2003, Diasio RB & Harris BE., 1989). The mode of action of 5-FU includes a series of enzyme activities showing differential expression/activity in drug resistant conditions. To improve the anticancer potential, modified drugs have been developed and additional drugs such as Capecitabine, Leucovorin (LV) and Methotrexate (MTX) are being used.

c. Capecitabine: To avoid DPD-mediated 5-FU degradation, pro-drugs have been developed. Capecitabine, an oral fluoropyrimidine, is readily absorbed through the gastrointestinal wall and converted to 5’-deoxy-5-fluorouridine (5’DFUR) in the liver by carboxylesterase followed by cytidine deaminase. Thymidine phosphorylase (TP) and/or uridinephosphorylase (UP) then convert 5’DFUR to 5-FU (Miwa, M. et al., 1998, Cao D. et al., 2002). Studies have shown Capecitabine to be significantly more active in tumors than in normal tissue (Ref) and demonstrating a significantly higher response rate than 5-FU (24.8% versus 15.5%) (Hoff PM. et al., 2001). However, there is not any improvement were seen in overall survival. Another limitation with Capecitabine is that it involves several intermediate enzymes activities that are modulated in drug resistant conditions.

d. Leucovorin (LV): LV is another chemotherapeutic drug used in colorectal cancer. It increases the intracellular concentration of the reduced folate CH2THF which is essential for binding FdUMP to TS. Co-administration of 5-FU and Leucovorin increases the concentration of CH2THF and has been shown to increase of 5-FU’s toxicity in vitro and in vivo (Matherly LH. et al., 1990, Park JG. et al., 1988, Nadal JC. et al., 1988, Wright JE. et al., 1989). LV enters the cell through the reduced folate carrier and is converted to CH2THF. As compared to 5-FU per se, the 5-FU/LV combination generates significantly higher response (11% versus 23%) but did not result in improvement in overall survival (Piedbois P. et al., 1992).

e. Methotrexate (MTX): MTX is an antifolate inhibitor of dihydrofolate reductase (DHFR), which catalyses conversion of dihydrofolate (DHF) to tetrahydrofolate (THF) (Gorlick R and Bertino J R., 1999). As THF is the precursor of CH2THF and is necessary
for dTMP synthesis, MTX inhibits both purine and thymidine biosynthesis. Several studies have reported that MTX pre-treatment significantly enhanced antitumor activity of 5-FU with increased formation of 5-FU ribonucleotides and increased 5-FU incorporation into RNA (Leyland-Jones B and O’Dwyer P J., 1986). Clinically, MTX and 5-FU combination was more potent than 5-FU alone for treatment of colorectal cancer with higher response rates (19% versus 10%) and overall survival (10.7 months versus 9.1 months) (Meta-analysis of randomized trials testing the biochemical modulation of fluorouracil by methotrexate in metastatic colorectal cancer-1994).

An important limitation of 5-FU is that even in combination with Capecitabine, Leucovorin and Methotrexate, there is very minor improvement in terms of its anticancer potential (enhanced from 11% to 24.8%, 23%, and 19% respectively) and the impact on overall survival has been negligible or minor (MTX combination yielded an increase of only 1.6 months while the other combinations did not impact overall survival). Another limitation of this drug is that tumors acquire resistance frequently to 5-FU and its combinations.

f. Oxaliplatin: Oxaliplatin (trans-/-diamino cyclo hexane oxalato platinum; LOHP) is a platinum derivative with two groups, oxalate and di amino cyclo hexane (DACH) of which oxalate acts as hydrolysable ligand and DACH acts as carrier (Figure 1.2). Oxalate was the 1st platinum derivative for the treatment of advanced colorectal cancer and was approved in France (Bleiberg H., 1998). As shown in figure 1.3 oxalate binds and cross-links strands of DNA, forming a DNA adduct thus inhibiting DNA replication and transcription (Kurniali PC. et al., 2010, De Gramont A. et al., 2000). Oxaliplatin has a DACH ring and thus has a different DNA binding than cisplatin. Cisplatin-DNA is recognized by mismatch repair system but the oxaliplatin-DNA adducts remains undetected (Yamada M. et al., 1997, Mello JA. et al., 1996). Cisplatin-DNA adduct elicits DNA repair mechanism which ultimately activates apoptosis when cell senses that repair is not possible. However, in case of defective mismatch repair, cisplatin-DNA adducts fails to activate apoptosis and results in resistance to cisplatin (Fink D. et al, 1996). In case of Oxaliplatin, the DACH ring of Oxaliplatin prevents binding of the mismatch repair molecules (Scheeff ED. et al.,
Oxaliplatin down-regulates TS and increases the effects of 5-FU when both drugs are used together (Longley DB. et al., 2003, Amatori F. et al., 2006).

Figure 1.3 Structure of DNA adduct formed by Cisplatin and Oxaliplatin.

Even though the mismatch repair system is unable to bind on oxaliplatin-DNA adduct due to the presence of bulky and hydrophobic group DACH, the DNA adduct may be removed by Nucleotide excision repair (NER) mechanism and cause resistance against oxaliplatin. Polymorphisms in some of NER coding gene may be involved in reducing the antitumor potential of oxaliplatin (Shen MR. et al., 1998, Park DJ. et al., 2001, Stoehlmacher J. et al., 2004, Yin M. et al., 2011, Lai JI. et al., 2009).

**g. Irinotecan:** Irinotecan is an analogue of Camptothesine obtained from a Chinese tree *Camptotheca acuminata* (Gerrits CJ. et al., 1997, Fujita K. et al., 2015). Irinotecan is used as a pro-drug which metabolizes into 7-ethyl-10-hydroxycamptothecine (SN-38). Irinotecan was first commercially available for the treatment of lung, cervical and ovarian cancer in Japan in 1994. It was approved for the treatment of metastatic CRC in the United State in 1996 as a single drug and after that in combination with 5-FU/leucovorine (Fujita K. et al., 2015). Irinotecan specifically inhibits the S-phase by interacting with the Topoisomerase (Topo I) - DNA complex (Liu LF. et al., 2000). Topoisomerases are involved in reducing the over-winding of DNA during transcription, replication and repair. They form nicks and then repair the phosphodiester bond of the DNA backbone. However, when the
irinotecan-Topo-1 complex collides with a replication fork, double stranded DNA breaks occur arresting the replication fork irreversibly and leading to cell death. Additionally, the collision prevents or delays G2 due to the DNA damage found during S-phase checkpoint (Liu LF. et al., 2000, Shao RG. et al., 1999). But irinotecan metabolizes inside the body and passes through many enzymatic processes and is finally converted to the more active metabolite SN-38. These metabolic activities involve carboxyl esterase enzyme and there is patient to patient variability in its expression leading to differential irinotecan sensitivity/resistance (Van Ark-Otte J. et al., 1998). The active metabolite SN-38 is further converted into an inactive form SN-38G by glucuronidation by UGT 1A1. UGT found over-expressed in case of irinotecan resistant lung cancer cells (Takahashi T. et al., 1997). P-glycoprotein and multidrug resistance-associated proteins (MRP) are over-expressed in resistant tumor cells and cause the efflux of SN-38 and irinotecan (Loe DW. et al., 1996, Chu XY. et al., 1999). Cell lines treated continuously with irinotecan have reduced sensitivity as well as reduced expression of Topo-1 suggesting that cytotoxic effects of irinotecan depends on Topo-1 expression level in tumor cells (Giovanella BC. et al., 1989).

After radiotherapy and chemotherapy, the drug discovery has been shifted towards more specific and targeted therapy. Targeted therapy includes monoclonal antibody based anticancer drugs (Immunotherapy) and small molecules based kinase inhibitor.

**iv. Targeted therapy**

Most of the targeted drugs are used to inhabit the kinase activity of surface associated receptors and intracellular molecules. Before discussion about targeted therapy, we would like to give some brief about Kinase enzymes.

**a. Basics of Kinase enzyme:** Kinase is a large group of enzyme which involved in mediating phosphate group transfer from high energy molecules such as ATP to substrate, process called phosphorylation. Based on nature of substrate, kinases are classified as Protein kinase, lipid kinase, carbohydrate kinase, nucleotide kinase etc. Protein kinase further classified based on target amino acid residue which is going to be phosphorylated. Protein kinase may be tyrosine kinase or serin-threonin kinase. Tyrosine kinase further sub-divided into receptor tyrosine kinase and non-receptor tyrosine kinase. Enzyme activity of protein kinases are under tight control and
involved in various cellular processes such as cell division, metabolism, movement, survival and apoptosis. Deregulation in the activity of these kinases may leads to tumor initiation and progression. Receptor Tyrosine Kinases (RTK) located on the cell surface as trans-membrane protein. As shown in figure 1.4 kinases have three different domains, an extracellular domain (Ligand binding domain), a trans-membrane domain, and an intracellular domain (Kinase domain). Intracellular domain consists of N-terminal lobe, ATP binding cleft, C-terminal lobe and Activation loop. ATP binding cleft have different sites like Adenine, Sugar and Phosphate binding site and another region that is hydrophobic region. Cytoplasmic tyrosine kinases are similar to receptor tyrosine kinases but lack the trans-membrane domain (Gotink KJ. and Verheul HM., 2010). About 518 kinases are reported in the human genome out of which 90 are tyrosine kinases, 388 are serin-threonin kinases and 40 are atypical kinases (Gotink KJ and Verheul HM., 2010, Zhang J. et al., 2009).

Figure 1.4 Structure of Receptor tyrosine kinase (RTK) and location of active site of the protein. RTK have three domains- extracellular domain, transmembrane domain and intracellular domain. Intracellular domain subdivided into N-terminal lobe, ATP binding cleft, C-terminal domain and activation loop. ATP-binding cleft have four different regions, phosphate binding region, adenosine binding region, sugar region and hydrophobic region.

Approximately 30 protein kinases are targeted to develop anticancer drugs which are at least in phase-1 clinical trial (Zhang J. et al., 2009). About 218 kinases genes are supposed to be altered during various pathological conditions like malignancy,
viral infection and other human diseases making them promising therapeutic targets (Manning G. et al., 2002).

b. Immunotherapy: Monoclonal antibody developed against VEGFR (Vascular endothelial growth factor receptor) (Bevacizumab) (Davidson M. et al., 2015) and EGFR (Epidermal growth factor receptor) (Cetuximab and Panitumumab) are used as anticancer drugs for treatment of various human cancers in advance stages including Colorectal Cancer (Chen CJ. et al., 2011). Bevacizumab is a humanized anti-VEGF-A monoclonal antibody. This is most extensively studied anti-cancer drug used for the treatment of advanced stage of colorectal cancer. VEGF is up-regulated in most of the human cancer and its expression is induced in hypoxic condition in solid tumors. Bevacizumab targets VEGF and blocks this pathway leading to tumor reduction in metastatic colon cancer (Saltz LB. et al., 2008). Panitumumab is completely human derived monoclonal antibody targeting extracellular domain of EGFR. Cetuximab is a mouse/human chimeric IgG1 immunoglobulin that also induces antibody dependent cytotoxicity through activation of the host immune response (Ciardiello F. and Tortora G., 2008). Both antibodies are used in combination with fluoropyrimidines, oxaliplatin or irinotecan for metastatic colorectal cancer (Chen CJ. et al., 2011, Van Cutsem E. et al., 2009, Douillard JY. et al., 2010) but the response depends on KRAS mutation status.

c. Synthetic small molecule based kinase Inhibitors

In a high throughput screen (HTS), 3-thienyl urea was identified as a reversible Raf kinase inhibitor. This finding shows that urea may inhibit kinases (Smith RA. et al., 2001). Chemical modifications on the lead compound, 3-thienyl urea, were done to increase its activity, resulting in the synthesis of the derivative, BAY 43-9006 (Sorafenib), a bis-aryl urea (Khire UR.et al., 2004) (Figure 1.5).

Sorafenib (BAY 43-9006): Sorafenib is the first multi-kinase inhibitor successfully developed and approved for the treatment of renal cell carcinoma and hepatocellular carcinoma. Journey of sorafenib discovery started in 1994 by Bayer and Onyx in collaboration to discover novel drugs targeting the Ras-Raf-MEK-ERK pathway. HTS screening for Raf1 kinase inhibitory activity was started in 1995, and testing of about 200,000 compounds (Wilhelm S. et al., 2007, Riedl B. et al., 2001, Smith RA. et al., 2001). Finally, 3-thienyl urea found as a promising lead compound,
with a Raf1 IC\textsubscript{50} of 17 μM. Further lead 3-thienyl urea was improved to IC\textsubscript{50} 1.7 μM by a 4-methyl substitution on the phenyl ring. However, no variants of this 3-thienyl urea with IC\textsubscript{50} values below 1 μM were identified while pursuing a traditional medicinal chemistry approach. A library of 1,000 bis-aryl urea analogues of the lead compound was then constructed, using rapid parallel synthesis techniques (Smith RA. et al., 2001). This combinatorial library was then screened against Raf1 to identify a new analogue, 3-amino-isoxazole (compound 3), exhibiting a Raf1 kinase IC\textsubscript{50} of 1.1 μM (Wilhelm S. et al., 2007, Lowinger TB. et al., 2002). From this point, the inhibitory potency of compound 3 was increased five fold (Raf1 kinase IC\textsubscript{50} 230 nM) by replacing its distal ring with a 4-pyridyl moiety which produced compound 4 (Lowinger TB. et al., 2002). Further SAR (Structure Activity Relationship) studies were undertaken. This work suggests that although the urea moiety was essential for Raf1 kinase inhibitory activity, aromatic replacements of the heterocyclic moiety of compound 4, resulting in diphenyl urea, were tolerated. Finally, modification of the distal pyridine ring while maintaining the diphenylureamo moiety, leads to the identification of Sorafenib (Figure 1.5) (Lowinger TB. et al., 2002, Wilhelm S. et al., 2001).

\[ \text{Sorafenib} \]

Figure 1.5 Structure of Raf-1 kinase inhibitors identified as 3-thienyl urea as compound 1 and others are modified compound with increased inhibitory effects and finally got Sorafenib and Regorafenib.

In vitro assays confirmed that Sorafenib is a potent in vitro inhibitor of Raf1 kinase (IC\textsubscript{50} of 6 nM) (Wilhelm SM. et al., 2004). Sorafenib was also shown inhibition to the
wild-type B-Raf, and oncogenic b-raf V600E serine/threonine kinases, RTKs (VEGFRs 1/2/3, PDGFRβ, FGFR1, c-Kit, Flt-3 and RET) (Wilhelm SM. et al., 2004, Carlomagno F. et al., 2006). Sorafenib is a big achievement in the field of drug discovery. Further modification in the structure of Sorafenib leads to identification of potentially more effective and broad range of kinases inhibitor that is Regorafenib with an extra F-atom in the central phenyl ring (Figure 1.5). 

Table 1.1 Sorafenib and Regorafenib target molecules and their respective IC_{50} value.

<table>
<thead>
<tr>
<th>Molecular target</th>
<th>Sorafenib IC_{50} (nM) ± SD</th>
<th>Regorafenib IC_{50} (nM) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-RAF</td>
<td>2.5±0.6</td>
<td>6±3</td>
</tr>
<tr>
<td>BRAF</td>
<td>28±10</td>
<td>22±6</td>
</tr>
<tr>
<td>BRAF\text{V600E}</td>
<td>19±6</td>
<td>38±9</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>13±0.4</td>
<td>NA</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>4.2±1.6^ {a}</td>
<td>90±15</td>
</tr>
<tr>
<td>VEGFR-3</td>
<td>46±10.8</td>
<td>20±6</td>
</tr>
<tr>
<td>TIE-2</td>
<td>311±46</td>
<td>NA</td>
</tr>
<tr>
<td>PDGFR-β</td>
<td>22±3</td>
<td>57±20</td>
</tr>
<tr>
<td>FGFR-1</td>
<td>202±18</td>
<td>580±100</td>
</tr>
<tr>
<td>C-Kit</td>
<td>7±2</td>
<td>68±21</td>
</tr>
<tr>
<td>RET</td>
<td>1.5±0.7</td>
<td>NA</td>
</tr>
<tr>
<td>Flt-3</td>
<td>NA</td>
<td>58±20</td>
</tr>
</tbody>
</table>

(Ravi S. et al., 2014)

Regorafenib (BAY 73–4506, Stivarga), a diphenylurea oral multikinase inhibitor, structurally comparable to sorafenib has recently been approved for the treatment of metastatic colorectal cancer and advanced gastrointestinal stromal tumors that are resistant to other therapies (Chu E., 2012). Multiple membrane-bound and intracellular kinases are inhibited by regorafenib and its active metabolites. Therefore, multiple normal cellular functions and pathologic processes are inhibited, including the RET, VEGFR1, VEGFR2, VEGFR3, KIT, PDGFR-alpha, PDGFR-beta, FGFR1, FGFR2, TIE2, DDR2, Trk2A, Eph2A, RAF-1, BRAF, BRAFV600E, SAPK2, PTK5, and Abl pathways (Cheng YD. et al., 2013, Wilhelm SM. et al., 2011, Aprile G. et al., 2013). Mutations in the KRAS gene, occurring in 40% of CRC patients, or BRAF mutations, which occur with a frequency of 5%-12%, leading to constitutive activation of the mitogen activated protein kinase (MAPK) pathway independent of the EGFR status (Davies H. et al., 2002, Andreyev HJN. et al., 2001). These are the main reasons for failure of many chemo-therapeutic and molecularly targeted drugs such as anti-EGFR and anti-VEGFR targeted drugs. Its ability in inhibiting the wild-type and mutant form of BRAF
protein has a great therapeutic implication in the management of mCRC with BRAF/V600E that fails to respond to cetuximab or EGFR therapy (Scott M. et al., 2011). Activity of regorafenib against the tumor proliferation and survival pathway (RAS/RAF/MAPK) and tumor vasculature pathway represents the basis of its high clinical efficacy. Regorafenib is particularly relevant for patients with mutations in the KRAS gene who are non-responsive to anti-EGFR treatments, which are commonly used agents in later lines of therapy. Regorafenib effectively inhibits growth of cell lines which showing resistance towards other drugs like Colo-205, HT-29 (Festino L. et al., 2013). The level of activated pERK1/2, detected by immuno-histochemistry, was strongly reduced in treated mice, indicating that, in vivo, Regorafenib effectively inhibited the RAF/MEK/ERK signaling cascade (Festino L. et al., 2013).

1.11 Limitations of available therapies

Despite significant progress in early cancer detection and systemic treatment of colorectal cancer (CRC), resistance to anti-cancer therapies is a major problem of current cancer research. Resistance to anti-cancer therapies is broadly categorized as intrinsic and acquired resistance. Intrinsic resistance indicates that before treatment, resistance-mediating factors exist in the tumor cells. Acquired resistance develops during treatment and tumor cells initially sensitive would later acquire resistance-mediating factors and become non responsive. In case of long term radiotherapy, the pathological response varied from potentially effective to completely resistant. It also varies from patient to patient (Rodel C. et al., 2005). It might be possible because the tumor micro-environment turns hypoxic which might not favor radiotherapy (Marian G. et al., 2012, Vaupel P. et al., 1991, Steel GG. et al., 1989). Cancer patients respond differently depending on individual genetic sensitivity (Steel GG. et al., 1979, Marian G. et al., 2012). Recent studies showed that irradiation enriches the fraction of cells expressing CSC marker suggest that radiation treatment may target mainly the bulk of the tumor rather than the cancer stem cells (CSCs) (Ezashi T. et al., 2005, Keith B. et al., 2007, Moeller BJ. et al., 2005). Chemotherapy is one of the principal modes of treatment for cancer, but the effectiveness of chemotherapy is limited by drug resistance. The mode of action of 5-FU and its
derivate capecitabine include a series of enzyme activity which are showing differential expression/activity in drug resistant condition. Oxaliplatin linked DNA-adducts may be removed by Nucleotide excision repair (NER) mechanism and cause resistance against oxaliplatin. Polymorphism have been identified in some of NER coding gene which may be involved in reducing the anti-tumor potential of oxaliplatin (Shen MR. et al., 1998, Park DJ. et. al., 2001, Stoehlmacher J. et al., 2004).

Active metabolite of Irinotecan, SN-38, is converted into inactive form SN-38G by glucuronidation by UGT 1A1. UGT is over-expressed in case of irinotecan resistant lung cancer cells (Takahashi T. et al., 1997). P-glycoprotein and Multidrug resistance proteins are over-expressed in resistance tumor cells to efflux SN-38 and irinotecan (Loe DW. et al., 1996, Chu XY. et al., 1999). Irinotecan cytotoxic effect depends on Topo-1 expression. In the resistant tumor cells, level of Topo-1 found reduced (Giovanella BC. et al., 1989). Resistance to anti-EGFR and VEGF monoclonal antibody occurs due to B-Raf and K-Ras gene mutations which are detected very frequently in colorectal cancer patients (Yokota T. et al., 2011, De Roock W. et al., 2010, Allegra CJ. et al., 2009). There are many literature reports that showed long-term exposure of cells to sorafenib leads to reduced sensitivity to the drug. PI3K/AKT, MAPK and JAK-STAT pathways were found more active in the sorafenib resistant tumor cells. In addition to this, EGFR and MDRP (multidrug resistance protein) molecules are also been reported in resistance development.

In case of Regorafenib such type of acquired resistance is not reported yet. Since Regorafenib being structurally and functionally similar to Sorafenib, there is a possibility to face the problem of resistance in future. It requires very extensive study to understand the exact mechanism and pathways involved in resistance development against these drugs. The aim of the study was “To understand the mechanisms involved in the resistance development against multi-Kinase inhibitors Sorafenib and Regorafenib.” To achieve this aim we have formulated the following objectives:

1. Development of drug resistant model cell line.
2. Study the alteration in different cell signaling pathways like JAK STAT, PI3K-AKT and MTOR etc.
3. Alteration in proteome and miRNA profiles and their involvement in attaining this drug resistance.

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