Chapter 1

Introduction
Introduction

1.0 Cancer

Cancer refers to a group of diseases characterized by the development of abnormal cells with uncontrolled cell division and the ability to infiltrate any tissue defying the principles of differentiation, denouncing the organization of the body and destroying normal tissue and transforming them to similar outrageous behavior. It is not a surprise to know that from the dawn of history people have been writing about cancer. The earliest evidence of cancer is found among fossilized bone tumors, and rectal cancer has also been diagnosed in an Ancient Egyptian mummy (1). The origin of the word cancer was credited to the Greek physician Hippocrates (460-370 BC), who was considered the “Father of Medicine.” Hippocrates used the terms carcinos and carcinoma (refer to a crab in Greek) to describe non-ulcer forming and ulcer-forming tumors. The Roman physician, Celsus (28-50 BC), later translated the Greek term into cancer, the Latin word for crab. Galen (130-200 AD), another Greek physician, used the word oncos (Greek for swelling) to describe tumors.

1.1 Types of Cancer

Cancers are often described by the body part in which they originated. However, for greater precision, cancers are classified by the type of cell from which the tumor cells originate. These types include:

- **Carcinoma**: Cancers derived from epithelial cells including those developing in the breast, prostate, lung, pancreas, and colon are referred to as carcinomas.
- **Sarcoma**: Cancers arising from connective tissue (i.e., bone, cartilage, fat, nerve), each of which develop from cells originating in mesenchymal cells outside the bone marrow.
- **Lymphoma and leukemia**: These are the cancers arise from blood cells.
- **Germ cell tumor**: Cancers derived from pluripotent cells, most often presenting in the testicle or the ovary.
- **Blastoma**: Cancers derived from immature "precursor" cells or embryonic tissue. Blastomas are more common in children than in older adults.
1.2 Anatomy of the colon and rectum

The colon and rectum make up the large intestine, which plays an important role in absorbing the fluids to form solid waste in the form of feces. The colon is divided into four sections; ascending, transverse, descending and sigmoid colons. The colon makes up the first five to six feet of the large intestine, and the rectum makes up the last six inches, ending as the anus (Fig 1).

![Fig 1: Anatomy of the gastrointestinal system.](http://lyceum.algonquincollege.com/lts/onlineCourses/anatomy/App_Themes/e-learning/images/diagrams/module13-3)

1.3 Colorectal cancer

Cancer that originates in the colon and rectum is called colorectal cancer (CRC) or more precisely it may also be referred to as colon cancer or rectal cancer and are in general, discussed together. It begins when normal cells in the lining of the colon or rectum transform and grows uncontrollably, forming a mass called a tumor. A tumor can be benign or malignant, where benign tumors will not spread or invade whereas, malignant tumors can spread to other parts of the body. CRC very often begins as a polyp, which is a noncancerous growth that may develop on the inner wall of the colon or rectum as people get older. If polyps are not treated, they may become adenomatous polyps, which can eventually progress into cancer. There are several types
of polyps. Adenomatous polyps, or adenomas, are growths that may become cancerous and can be found with a colonoscopy. Polyps are most easily detected during colonoscopy because they usually bulge into the colon, forming a mass on the wall of the colon (Fig 2). About 10% of colon polyps are flat and hard to find with a colonoscopy, unless a dye is used to highlight them. These flat polyps have a high risk of becoming cancerous, regardless of their size.

![Colon and polyp image](image-url)

**Fig 2:** Incidence of CRC in various parts of colon and colonoscopy view of polyp

(Mayo foundation for medical education and research)

### 1.4 Statistics of colorectal cancer

#### 1.4.1 New cases:
CRC is the third most common cancer in both men and women with significant geographical, racial and ethnic variation in its pattern and rate of incidence. According to the American Cancer Society, an estimated 95,520 new cases of colon cancer and 39,910 new cases of rectal cancer are expected to be diagnosed in 2017 in USA. The burden of CRC in India has been rising slowly over many decades and rank 9th may be due to economical transition and changing life styles.

#### 1.4.2 Incidence:
Rates of CRC incidence vary markedly worldwide. Highest rates of incidence of CRC were noticed in Europe, North America, and Oceania. In contrast, the lowest rates were observed from registries in Asia, Africa, and South America. Incidence and death rates for CRC increase with age. Overall, 90% of new cases and 93% of deaths occur in people of 50 years (yrs) and older (2). The median age at which colon cancer has been diagnosed is 69 yrs in men.
and 73 yrs in women and for rectal cancer it is 63 yrs in men and 65 yrs in women (3). Overall, CRC incidence and mortality rates are about 30% to 40% higher in men than in women probably, because of gender-related differences in exposure to hormones and risk factors. A decline (>4% per year) in the incidence of CRC was observed during the past two decades, due to changes in risk factors and the uptake of CRC screening among adults 50 years and older, however, it is increased by 1.8% per year among adults younger than age 50 yrs (3, 4).

1.4.3 Deaths: An estimated 49,700 deaths from CRC are expected to occur in 2015 (5). CRC is the third leading cause of cancer death in both men and women and the second leading cause of cancer death when men and women are combined. Similar to incidence patterns, mortality rates are also declined most rapidly in the past decade (3% per year in both men and women) (3) due to improvements in treatment (12%), changes in risk factors (35%), and screening (53%) (4).

1.4.4 Survival: The relative rate of survival for CRC is 65% at 5 yrs following diagnosis and 58% at 10 yrs. When CRC is detected at a localized stage, the 5-year survival is 90% (only 40% of CRCs are diagnosed at this early stage) and if the cancer has spread to involve nearby organs or lymph nodes, the 5-year survival drops to 71% and the 5-year survival is only 13% if the disease has spread to distant organs. Rectal cancer is diagnosed at a localized stage more often than colon cancer, which probably contributes to the higher overall survival for rectal cancer (3). As of January 2012, there were almost 1.2 million Americans alive with a history of CRC (6). The survival disparities may be attributed to access for early detection tests, receipt of timely and high-quality treatment, and the prevalence of co-morbidities (7).

1.4.5 Indian scenario: The annual incidence rates (AAR) for colon cancer and rectal cancer in men are 4.4 and 4.1 per 100,000 respectively. The AAR for colon cancer in women is 3.9 per 100,000. The incidence of CRC in India is lower compared to western countries, colon and rectal cancers ranks 8th and 9th position respectively in men in India (8). Several studies have shown that a relatively high proportion of young age rectal cancer (RC), with a mean age of around 40-45 yrs from India and Bangladesh (9). Majority of the reports on young age RC or CRC in India came from West Bengal which has similar ethnic, linguistic, dietary, cultural and social characteristics like Bangladesh (10).
1.5 Types of colorectal cancers

There are several types of cancers that arise in the colon or rectum.

- **Adenocarcinoma**: These represent more than 95% of colon and rectal cancers. It typically starts within the intestinal glandular cells (mucus secreting) that line the inside of the colon and rectum (“Adeno” is the prefix for gland) and spreads deeper to other layers. There are two main subtypes of adenocarcinoma:
  - **Mucinous adenocarcinoma** account for 10% to 15% of all CRC adenocarcinomas and spread faster (more aggressive) due to high mucus content.
  - **Signet ring cell adenocarcinoma** accounts for less than 1% and so named for its appearance under a microscope. It is typically aggressive and may be difficult to treat.

Apart from the above mentioned types, there are few other types of CRCs which are rare and account for 5% of all cases.

1.6 Development of CRC

Tumorigenesis is a collective process involving a cascade of genetic events resulting in activation of oncogenes and inactivation of tumor suppressor genes, which allow escape from the tight constraints that control normal cells. Most CRCs are thought to develop through an orderly series of events known as adenoma carcinoma sequence, where, normal colonic mucosa is transformed into adenoma, which then transforms into adenocarcinoma (Fig 3) (11). In 1990, Fearon and Vogelstein (12) described the molecular basis of CRC as a multistep process that requires germ line and somatic mutations for malignant transformation. The APC (adenomatous polyposis coli) gene is a negative regulator of β-catenin and is considered to be the “gatekeeper” in the adenoma to carcinoma sequence. Inactivation of APC gene results in increased nuclear accumulation of β-catenin for transcriptional activation. Mutations in the APC gene occur early in the development of CRC as is seen in aberrant crypt foci (ACF), which are the earliest malignant lesions, considered to be precursors for adenoma and carcinoma of the colon (13). The exact sequence of commonly acquired genetic changes accumulated subsequent to inactivation of APC is variable. K-ras mutations are found in ~50% of CRC and are thought to be relatively early events that correlate histologically with early to late adenomas. There is good evidence to
suggest that p53 mutations occur more frequently in high-grade dysplastic polyps and are thought to mark the transition from adenoma to carcinoma (14).

There are two pathways for CRC development. 1) Chromosomal instability, which is characterized by loss of heterozygosity, is responsible for 80–85% of sporadic colorectal adenomas and carcinomas; and 2) Microsatellite instability (MSI), which is responsible for 15%–20% of sporadic CRCs (15). MSI result from mutations in one of the DNA mismatch repair genes (MMR). CRC may arise through a third genetically and clinically distinct pathway against a background of ulcerative colitis known as ulcerative colitis associated colorectal carcinomas.

![Molecular events/alterations occur during adenoma to carcinoma sequence](image)

**Fig 3:** Molecular events/alterations occur during adenoma to carcinoma sequence (16).

### 1.7 Stages of colorectal cancer

The extent to which cancer has spread from its site of origin in the colon to the liver, lungs or other organs in the abdominal cavity through blood and lymph vessels (metastasis) at the time of diagnosis is described as its stage (Fig 4). Staging is essential in determining the treatment options and for prognostic evaluation. More than one system is used for staging of the cancer. The two most common staging systems are the TNM (tumor, lymph node and metastasis) system typically used in clinical settings and the Surveillance, Epidemiology, and End Results summary staging system, used for descriptive and statistical analysis of tumor registry data.
The TNM staging system is one of the most commonly used systems which replace the Dukes classification proposed in 1929-35 (17). It is based on the extent of the tumor (T), the extent of spread to the lymph nodes (N), and the presence of metastasis (M).

![Fig 4: Schematic illustration of stages of CRC (https://goo.gl/images/cucFxs)](https://goo.gl/images/cucFxs)

1.8 Risk factors associated with Colorectal Cancer

The several risk factors known to influence the development of CRC are shown in Table 1.

**Table 1: Risk factors associated with development of CRC**

| Known Risk Factors | • Personal history of adenomatous polyps  
| | • Personal and/or family history of CRC  
| | • Inflammatory Bowel Disease  
| | • Irritable Bowel Syndrome  
| | • Ulcerative colitis  
| Possible Risk Factors | • Environment  
| | • Diet  
| | • Tobacco use  
| | • Age over 50 yrs- at increased risk  
| | • Physical inactivity  

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1.9 Signs and Symptoms

Early stage CRC typically does not have any symptoms, however, people with CRC may experience the following signs and symptoms.

- A change in bowel habits
- Diarrhea, constipation, or feeling that the bowel does not empty completely
- Bright red or very dark blood in the stool
- Stools that look narrower or thinner than normal
- Discomfort in the abdomen, including frequent gas pains, bloating, fullness, and cramps
- Decreased appetite, or weight loss
- Weakness or fatigue
- Unexplained iron-deficiency anemia, resulting in a low number of red blood cells

1.10 Screening

Early screening reduces CRC mortality by decreasing the incidence of disease and also increasing the chances of survival by early detection and treatment. CRC usually does not have symptoms until the disease is advanced; therefore, regular screening beginning at the age of 50 yrs or even earlier if the people have any of the following risk factors.

- A personal history of CRC or adenomatous polyps
- A strong family history of CRC or polyps in a first-degree relative younger than 60 yrs or in two first-degree relatives of any age
- A personal history of chronic inflammatory bowel disease
- A family history of any hereditary CRC syndrome

1.10.1 Tests for detection of both adenomatous polyps and cancer

Flexible Sigmoidoscopy- It is used to examine the rectum and lower part of colon for presence of polyps, cancer, and other abnormalities and it is associated to reduce CRC incidence and mortality by 21% and 26% respectively (18).
Colonoscopy- Colonoscopy is the most sensitive method for the detection of CRC or polyps. Patients who had adenomas removed during colonoscopy (with follow-up colonoscopy at one or three yrs) had a 53% lower risk of death from CRC than the general population (19).

Computed tomography (CT) colonography- It is less invasive, requires no recovery time, and takes approximately, 10 to 15 min to complete. Patients with polyps of significant size (>5 mm) or other abnormal results are referred for colonoscopy.

Double contrast barium enema (DCBE) - For patients who cannot have a colonoscopy, an enema containing barium is given, which helps the outline of the colon and rectum stand out on x-rays. This method is less sensitive than colonoscopy for visualizing small polyps or cancers.

1.10.2 Tests for detecting colorectal cancer

Although high-sensitivity stool tests will detect some precancerous polyps, the potential for prevention is limited and cannot be the primary goal of screening with these tests.

Fecal occult blood test - This is a test used to find blood in the feces, which can be a sign of polyps or cancer and the test may be positive even in many other conditions including bleeding in the stomach or upper GI tract.

Stool DNA tests- It is based on the detection of altered DNA from cells that are shed from polyps and cancers into the stool.

While there are plenty of such tests available, the routine schedule for screening tests for average risk adults includes:

- Flexible sigmoidoscopy- every five yrs
- Colonoscopy- every ten yrs
- DCBE- every five yrs
- CT colonography- every five yrs

It is important to note, regardless of the screening test and schedule, any test that indicates an abnormality should be followed up with a colonoscopy for complete diagnostic evaluation.
1.11 Prevention of colorectal cancer

The slow course of growth from precancerous polyp to invasive cancer provides a unique opportunity for the prevention and early detection of CRC. In addition, long-term, regular use of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) (20), postmenopausal hormones (21) and diets rich in vegetables and high fiber may help to lower the risk of CRC. However, NSAIDs and hormones are associated with side effects of heart attack and breast cancers respectively.

1.12 Colorectal cancer treatment

In general, cancer care or treatment plan is designed by multidisciplinary teams. Currently, multiple therapies that are available for CRC treatment include, surgery, radiotherapy, hormone therapy and combination of chemotherapy. Although, the indications to use a specific type of therapy vary due to histopathologic characteristics of the tumor, stage of cancer, possible side effects, the best results are often obtained, when these treatment methods are applied in combination.

1.12.1 Surgery

The goal of surgery is to remove colorectal tumors, as well as a margin of surrounding healthy tissue and several nearby lymph nodes to ensure that all of the cancerous tissue is removed. For many patients, surgery will be combined with other treatments, such as chemotherapy, radiation therapy or hormone therapy. These nonsurgical treatments may be administered before or after surgery to help prevent cancer growth, spread or recurrence.

1.12.2 Radiation therapy

Radiation therapy is the use of high-energy x-rays to destroy cancer cells or shrink tumors. Since each cancer type requires a different approach, radiation therapy may be used alone or in combination with other treatments. Radiation treatment for CRC requires accuracy and precision. With advances in radiation therapy delivery systems, it is able to target difficult-to-reach tumors
and direct higher radiation doses at CRC cells, while reducing exposure to normal, healthy tissue.

1.12.3 Chemotherapy

Chemotherapy is the use of drugs to destroy cancer cells and their ability to regenerate. In some cases of rectal cancer, it may be given before surgery (neo adjuvant chemotherapy) to shrink the size of the tumor, so that it is easy to remove. Chemotherapy is often given at the same time as radiation therapy, called chemoradiation therapy to increase the efficacy of the radiation therapy. Chemotherapy given after surgery is known as adjuvant therapy to reduce the chance of cancer recurrence by eliminating cancer cells that are infiltrated into circulation or surrounding tissues. Currently, several drugs that are approved by the U.S. Food and Drug Administration (FDA) for CRC treatments are as follows;

- Fluorouracil (5-FU, Adrucil) is a fluorinated pyrimidine that is considered as the backbone for CRC treatment. It acts primarily by inhibiting thymidylate synthase, the rate-limiting enzyme in de novo pyrimidine nucleotide synthesis (22). Fluorouracil is usually administered with leucovorin, a reduced folate, which stabilizes the binding of fluorouracil to thymidylate synthase, thereby, enhancing the inhibition of DNA synthesis (23).
- Capecitabine (Xeloda) is also a prodrug of fluorouracil used for oral administration (24).
- Irinotecan (Camptosar, also known as CPT-11) is a semisynthetic derivative of the natural alkaloid camptothecin. Its cytotoxic effect is mediated through its interaction with the enzyme topoisomerase I, which causes single stranded DNA breaks (25).
- Oxaliplatin (Eloxatin) is another promising drug in the treatment of CRC, belongs to third generation platinum derivative. It forms DNA adducts and induces cellular apoptosis (26) and shown to be highly synergistic with fluorouracil.

Although these drugs hold mainstay in the treatment of CRC, due to lack of specificity they are often associated with general side effects such as bone marrow depression, neuropathy and GI symptoms. However medications to manage these side effects are available.
Hyperthermic intraperitoneal chemotherapy is a highly concentrated, heated (41-42° C) chemotherapy treatment that is delivered directly to destroy microscopic cancer cells that remain in the abdomen after surgery and have not spread to organs such as liver or lungs, or to lymph nodes outside of the abdominal cavity. The advantage of this method is high doses of chemotherapy with improved absorption and fewer side effects by minimizing the exposure to other tissue (27).

1.12.4 Targeted therapy

Although chemotherapeutic agents used in current clinical practice have played a significant role in reducing mortality/morbidity, often lead to have undesirable side effects. Recent improvements in understanding of molecular mechanisms that control tumor cell proliferation, motility, invasion and metastasis have led to the identification of important therapeutic targets. Targeted therapy could be defined as a drug with a distinct mechanism that specifically inactivate cancer specific genes, proteins or biological pathway consequently regression or destruction of the malignant process. As these markers are unique for each tumor type, it is essential to perform tests to identify the targets before selecting the suitable regimen. There are two main categories of targeted therapy drugs such as; small molecules and antibody based therapies (27).

Currently, two promising classes of targeted compounds have been introduced into the clinical management of advanced CRC: Cetuximab and panitumumab are mouse and human monoclonal antibodies respectively against epidermal growth factor receptor (EGFR) signaling pathway (28). The EGFR is a transmembrane glycoprotein that is involved in cellular growth, differentiation, proliferation, and programmed cell death and over expressed in certain tumors. However, as per FDA recommendations, these drugs are reserved for patients whose tumors express wild type ras. Similarly, bevacizumab is a human monoclonal antibody directed against the vascular endothelial growth factor (VEGF), has been in clinical use to prevent angiogenesis (29). These drugs are often able to attack cancer cells selectively while causing minimal damage to normal cells or tissue. However, the side effects are less severe compared to cytotoxic drugs.
1.12.5 Combined therapy

As most of the cancers develop due to multiple genetic alterations or abnormalities, it is seldom possible to cure cancer with single drug. Combination therapy has been the standard of care, in cancer treatment, since it is a rationale strategy for successful chemotherapy. However, identifying rational combination that leads to cancer cure is also an active area of research currently. This approach offers the integrated therapeutic benefits such as improved clinical outcome, overcome drug resistance and also there is a possibility to minimize systemic toxicity through the delivery of lower drug doses (30, 31). Improved patient compliance due to the reduced number of administrations is also an additional advantage of combination chemotherapy (32). However, there are some fundamental principles followed in combination chemotherapy such as, 1) use of drugs with non-overlapping toxicities so that each drug can be administered at near-maximal dose, 2) combine agents with different mechanisms of action and minimal cross-resistance in order to inhibit the emergence of broad spectrum drug resistance, 3) preferentially, use drugs with proven activity as single drugs and 4) administer the combination at early stage disease and at a schedule with a minimal treatment-free period between cycles but still allowing the recovery of sensitive target tissues (30, 31).

In fact, there are several molecular and pharmacological factors that determine the effectiveness of drug combinations and the “one-size fits all” theory in cancer treatment; especially, in combination treatment is not possible. Therefore, before opting for combination chemotherapy, a variety of factors should be taken into consideration including patient age, overall performance, genetic disposition, co morbidity, tumor characteristics, and pharmacoeconomics (33). The effect of drug pharmacokinetics on the combination should also be considered as drugs with very different half-lives have different interactions at different time points (34).

1.12.6 Anticancer agents

Anticancer agents may grossly be divided into two categories based on their source of production: natural or synthetic. Natural compounds with anticancer activity are better alternatives to chemotherapeutic agents because of their pleotropic activity and minimal side effects. According to the World Health Organization estimates, more than 80% of the world’s...
population still depends on herbal medicine for primary health care. These drugs are used either alone or in combination with other chemotherapeutic drugs in the treatment of lymphomas, leukemias, breast, testicular, lung cancers, and Kaposi’s sarcoma. There are many natural products and their derivatives have been identified as potent anticancer agents and currently used in clinical management of various cancer types. The clinical utility of these drugs is expanding and currently used against a variety of cancers including breast, ovarian and non-small-cell lung cancer. Another important addition to the anticancer arsenal is the class of clinically active camptothecin derivatives (topotecan for ovarian and small-cell lung cancers and irinotecan for CRCs) isolated from the Chinese ornamental tree, *Camptotheca acuminata* Decne (Nyssaceae). Although there are plenty of anticancer drugs from natural or synthetic source, the present study is designed to investigate the anticancer activity of limonene (natural) and BEZ-235 (synthetic). Hence, further description is confined only to these two drugs.

1.12.7 Limonene

D-limonene (1-methyl-4-(1-methylethenyl) cyclohexane) is a monocyclic monoterpen with a lemon-like odor and is a major constituent in several citrus oils (orange, lemon, mandarin, lime, and grapefruit). It is listed in the Code of Federal Regulation as generally recognized as safe and widely used as flavoring and fragrance additive in food industry (35). Limonene is colorless liquid with a molecular weight of 136.24 (molecular formula: C$_{10}$H$_{16}$; CAS-No. 5989-27-5) (Fig 5).

![Fig 5: Chemical structure of Limonene](image)

It is increasingly used as an industrial solvent in the manufacturing of resins, wetting and dispersing agent and in insect control. Therefore occupational exposure to limonene is an inevitable event during its production and use (36). The average daily dietary intake of limonene has been estimated to be about 0.3 mg/kg body weight (37). After oral administration it is
completely absorbed from GI tract (38) and rapidly distributed in various tissues but high concentrations are accumulated in fatty tissues such as adipose tissue and mammary gland than in less fatty tissues (39). Therefore, limonene and many of its clinically active metabolites such as perillic acid and dihydroperillic acid are considered as most promising agents for breast cancer studies. In phase I/II clinical trials, limonene has been shown to be highly tolerable and safe after single and repeated dosing at 0.5-8 g/m²/day for up to one year and also did not show any mutagenic, carcinogenic, or nephrotoxic effects (40).

Limonene is shown to have wide clinical applications in cancer as well as in other disease conditions. It is used to dissolve cholesterol containing gallstones (41) and due to its potential for neutralizing gastric acid, it has also been used to relieve heartburn and gastroesophageal reflux disorder (42). In preclinical cancer models, it has been shown to prevent or delay the growth of a number of cancer types including lymphomas (43), mammary (44), gastric (45), liver (46), lung (47) and prostate cancer (48).

Mechanism of action

The anticancer mechanism of limonene could be multifactorial. As most of the in vitro and in vivo studies demonstrated that the chemotherapeutic activity of limonene could be attributed to the induction of apoptosis. Ji et al found that limonene induced apoptotic cell death in leukemia cancer cells involves BAX expression, release of cytochrome c and activation of caspase suggesting that mitochondria mediated intrinsic death pathway may play a major role in the mechanism of limonene (49). Almost similar findings have been reported in LS147T CRC cells with concomitant decrease of PI3K/AKT phosphorylation resulting in apoptosis (50) and limonene also sensitizes the effect of docetaxel in human prostate cancer cells by generation of reactive oxygen species and induction of apoptosis through modulation of mitochondrial death pathway components (48).

1.12.8 NVP BEZ-235

NVP BEZ-235 (4-[2,3-dihydro-3methyl-2-oxo-8-(3-quinolinyl)-1H-imidazo[4,5-c]quinolin-1-yl]-α,α-dimethyl-benzeneacetonitrile) is a synthetic low molecular mass compound (molecular formula: C₃₀H₂₅N₅O; MW: 469.5; CAS-No. 915019-65-7) belonging to the class of
imidazoquinolines (Fig 6). It is supplied as a white crystalline solid, soluble in organic solvents like chloroform or dimethylformamide. Commercially it is also known as Dactolisib.

![Chemical structure of BEZ-235](image)

**Fig 6: Chemical structure of BEZ-235**

**Mechanism of action**

BEZ-235 is well tolerated after oral administration and enhances the efficacy of other chemotherapeutic drugs when tested in combination studies. Its synergistic anticancer effects have already been demonstrated in various combinations. It is a potent dual inhibitor of class 1 PI3K and mTORC1 and mTORC2 catalytic activity by competing at its ATP-binding site. While it inhibits PI3K isoforms and mutants at lower concentrations (nM), BEZ also shown to inhibit VEGF induced angiogenesis. In many clinical and preclinical cancer studies, BEZ is shown to inhibit the various types of cancers such as colon (51, 52, 53), breast (54), gastrointestinal (55) and glioma stem cells (56). Results from several *in vitro* and tumor xenograft mouse models also indicated the efficacy of BEZ against broad range of cancer cells with wild and mutant phenotypes.

There are varied mechanisms attributed for its clinical efficacy including induction of apoptosis, autophagy, impair DNA repair mechanisms, increase of radiosensitivity and cell cycle arrest. Although several PI3K signaling inhibitors are available, NVP BEZ-235 is selected based on the observation that drugs targeting both class IA PI3Ks and the catalytic site of mTOR are more effective (Fig 7). While the anticancer activity of BEZ has been well described, several of preclinical cancer studies, however, noticed a reversal of PI3K activity after prolonged treatment at low concentrations of BEZ (54), rendering the cells less sensitive to apoptosis. Therefore, it is suggested to use in combination with other agents to be more efficacious in cancer treatment.
**Fig 7:** Mechanism of action of BEZ-235 in PI3K signaling (modified from Nature reviews/cancer)

1.13 Biomarkers in colon cancer

Although clinicopathologic staging of CRC remains the standard for assessing patient risk and treatment protocols, a variety of biomarkers are being investigated for prognostic and predictive utility in CRC such as microsatellite instability (57), loss of heterozygosity 18q (58), VEGF, BRAF (59,60) and many of such markers are under evaluation for their clinical utility in the assessment of CRC.

1.14 Ras signaling pathway in CRC

The ras family of GTPases (H-ras, K-ras and N-ras) are proto-oncogene products that are critical components of signaling pathways leading from cell-surface receptors to the control of cellular proliferation, differentiation, or cell death. Since defects in ras signaling may result in malignant transformation, the activation of ras proteins is tightly controlled in normal cells.

Ras proteins function as GDP/GTP-regulated molecular switches that cycle between two conformational states: binding to GTP, the active form; and binding to GDP, the inactive form. Following activation by external signals, ras interacts with wide range of downstream signaling pathways, of which the RAF-MEK-ERK mediated mitogenic signaling pathway is well characterized (61). Phosphatidylinositol 3-kinases (PI3K) pathway is another well-characterized
ras effector family. Activation of PI3K and its downstream effectors mediate cell growth, cell cycle entry and cell survival (62). Due to their essential role in normal physiological process of cell growth, it is now clear that activating mutations in members of the ras family of genes are among the most common genetic alterations in human tumors (63). These mutations lock ras proteins into a constitutively GTP-bound and keep them in activated state with constant signaling of downstream effectors, even in the absence of upstream signaling by RTKs (receptor tyrosine kinase). About 30% of human cancers are known to have ras mutations, but highest incidence is noticed in pancreas (90%), colon (50%), thyroid (50%), lung (30%) and melanoma (25%) cancers (64).

1.14.1 K-ras mutations in colorectal cancer

The K-ras protein, also called p21, is a member of the ras super family of proteins (64). Point mutations in K-ras gene are believed to be among the earliest events in colorectal tumorigenesis found in about 35-45% of CRCs (65, 66). It has been detected in aberrant crypt foci, which are considered the first identifiable precursor lesion of CRC. The codons 12 and 13 (participate in the GTP binding domain of the protein) are two hotspots, which account for about 95% of all mutation types, with approximately, 80% and 15% occurring in codons 12 and 13 respectively.

The role of K-ras mutations as a prognostic marker in CRC patients is not conclusive, as it is limited only to a glycine to valine mutation (codon 12), which has a significant impact on failure-free survival and overall survival (67). It is also noted that CRC patients with both K-ras and BRAF- wild type and MSI-H exhibited the most favorable disease specific survival, whereas, the prognosis is poor in patients with mutated K-ras or BRAF and microsatellite stable than other groups of these three markers (68). However, K-ras mutation status has emerged as a predictive marker to identify patients with metastatic CRC that may benefit from EGFR inhibitors. Because K-ras is the most frequently mutated factor downstream of the EGFR signaling pathway, it is considered as a candidate molecular biomarker for anti-EGFR therapy. K-ras mutations activate the EGFR signaling pathway independent of receptor status, bypassing the efficacy of anti-EGFR therapy. Hence, it is very unlikely that CRC patients with K-ras mutations will benefit from anti-EGFR treatment such as cetuximab and panitumumab. Therefore, testing for K-ras mutations became an inevitable clinical practice in order to guide
treatment and it is vital that the benefits of adjuvant therapy with anti-EGFR drugs are limited to patients with K-ras wild type disease (69).

1.15 Role of Phosphatidylinositol 3-kinase (PIK3) signaling in colorectal cancer

The Phosphatidylinositol 3-kinases (PI3K) are heterodimeric lipid kinases composed of catalytic (p110α) and regulatory subunit (p85) variants and key regulators of cell growth, transformation, adhesion, apoptosis, survival and motility (70). The PI3K family of enzymes is divided into 3 main classes (class I, II, and III) of which class I PI3K is the best characterized and clearly implicated in human cancer (71). The catalytic subunits for the class I PI3Ks are p110α, p110β, p110γ, and p110δ which are the products of genes PIK3CA, PIK3CB, PIK3CG, and PIK3CD, respectively (72).

Activation of PI3-kinase by extracellular (RTK) signals leads to phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) on the plasma membrane to generate the second messenger, phosphatidylinositol-3,4,5-trisphosphate (PIP3). The growth signals initiated by these lipid products are exerted via its downstream components such as AKT, mTOR and p70 S6 kinase (73). Among these, AKT is a critical downstream effector of the PI3K pathway that is activated by phosphorylation at Thr308 and Ser473 by PDK1 and mTORC2 respectively (Fig 8). It regulates a number of critical cellular pathways leading to proliferation and inhibition of apoptosis (74). Phosphatase and tensin homolog (PTEN) is a tumor suppressor gene that encodes for a lipid phosphatase protein that down-regulates PI3K activity. The phosphatase activity of PTEN inhibits the AKT signaling pathway and affects regulation of the cell cycle. Mutations in this gene are also associated with a variety of cancers, including prostate, brain, skin, and breast (75).

PIK3CA gene (class I PI3K) alterations particularly somatic missense mutations were reported in many cancer types but colorectal, brain and gastric cancers were detected to have a high rate of mutation with frequencies of 32%, 27% and 25%, respectively (76). These mutations were shown to increase kinase activity of PIK3CA contributing to cellular transformation. Recent data also indicated that more than 80% of these mutations in PIK3CA cluster in two small conserved regions within the helical and kinase domains and were considered as hotspots (76). Though
these mutations were scattered across most of the exons, they were predominantly found in exon 9 (E542K and E545K) related to the helical domain of the protein; and exon 20 (H1047R) corresponding to the kinase domain. However, apparently, these two classes of PIK3CA mutations promote constitutive PI3K signaling through distinct mechanisms.

1.15.1 Targeting PI3K/AKT signaling pathway

With this backdrop, of late, the PI3K signaling pathway has become an attractive therapeutic target for cancer drug development. Several small molecules that inhibit the PI3K-AKT signaling pathway are in clinical development. Currently, four main classes of inhibitors are available such as AKT inhibitors, PI3K inhibitors, mTOR inhibitors and dual PI3K-mTOR inhibitors.

The use of PI3K inhibitors vs. dual PI3K-mTOR inhibitors largely depends on whether PI3K inhibition alone can down regulate mTORC1 signaling in a particular cancer type that is being treated. For example, there are cancers in which PI3K-AKT is the strongest input for mTORC1 activation such as tumors with PIK3CA mutations or loss of PTEN. In these cases, it may be rational to use specific PI3K inhibitors, which would not only down regulate mTORC1 signaling but also avoid side effects due to mTORC1 and mTORC2 inhibition. However, the use of dual PI3K-mTOR inhibitors might be beneficial in some cancers with BRAF or K-ras mutations, in which mTORC1 activity is not exclusively regulated by PI3K–AKT signaling (77). Though there are many AKT inhibitors in clinical development, the existence of AKT-independent effectors of PI3K signaling in a subset of cancers with PIK3CA mutations, might significantly affect the clinical efficacy of AKT inhibitors (78). The identification of biomarkers that predict sensitivity or resistance to PI3K inhibitors is extremely useful, because, there is a growing evidence that cancers with K-ras mutations are unlikely to be sensitive to single-agent PI3K inhibitors. In these cases, the combination of PI3K inhibitors with other agents that target additional pathways are predicted to be more beneficial (79).

1.15.2 mTOR in CRC

mTOR is a serine/threonine kinase that belongs to the PI3K-related protein kinase family and exists as two functionally distinct protein complexes: mTOR complex 1 and 2 (mTORC1 and
mTORC2). mTORC1 is activated by the PI3K/AKT pathway and is inhibited by the tuberous sclerosis 1 (TSC1)-TSC2 complex. It is a major regulator of ribosomal biogenesis and protein synthesis (80), through the phosphorylation and activation of S6K, and the phosphorylation and inactivation of the repressor of mRNA translation 4EBP1. Since they are the best characterized downstream targets of mTOR, the phosphorylation status of S6K and 4EBP1 are commonly used to evaluate mTORC1 activity. On the other hand, mTORC2, activated by growth factors, phosphorylates PKC-α, AKT (on Ser473) and regulates the activity of the small GTPases: Rac and Rho related to cell survival, migration and regulation of the actin cytoskeleton (81).

Therefore, mTOR pathway has also become an important target for cancer therapy. Based on their mechanism of action, mTOR inhibitors are grouped into two classes: 1) rapamycin and rapamycin analogues (rapalogs) are allosteric inhibitors of mTORC1. 2) There is a novel class of the small molecules that are mTOR kinase inhibitors. Indeed, rapamycin and its analogues (tmelisirolimus, everolimus and deforolimus) block only certain functions of mTORC1 and have no effects on mTORC2 (82). However, these novel kinase inhibitors bind to the ATP-binding site of mTOR and inhibit the catalytic activity of mTORC1 and mTORC2. They are reported as having more potent anticancer effects than rapamycin analogues. While initial studies using rapamycin to target mTOR, recent findings have demonstrated that inhibition of mTORC1 by rapalogs results in the activation of proliferative and survival signals such as PI3K/AKT signaling pathways through the removal of a negative feedback loop (83). Therefore, it is hypothesized that the use of dual PI3K-mTOR inhibitors might alleviate this feedback activation of PI3K signaling and yield greater therapeutic benefit (84). They may be effective even in cancers with AKT mutations, because both PI3K and mTORC2 activity are required for complete activation of AKT.
1.16 Cell cycle and cancer

A series of coordinated events that take place in a cell leading to its division and duplication of DNA in a semi-conservative manner to produce two daughter cells is known as “cell division cycle”. In general, the cell cycle starts with the G_0 phase (resting/quiescent/senescent phase), where the cell has left the cycle and stop dividing. Non-proliferative and fully differentiated cells (such as neurons) in multicellular eukaryotes normally enter the quiescent G_0 state from G_1 and may remain quiescent for long periods of time, possibly indefinitely.

The cell cycle is divided into three phases: interphase, the mitotic (M) phase and cytokinesis (Fig 9). Interphase is also known as preparatory phase during which a cell grows and accumulates nutrients for cell division to proceed through the stages of G_1, S and G_2, followed by the cycle of mitosis and cytokinesis. Typically the interphase may constitute about 90% of the total time required for the cell cycle. During G_1 (gap 1) phase the cell integrates growth inhibitory and mitogenic signals and either proceeds towards S phase or exit the cell cycle. S phase stands for synthesis and is the stage during which DNA is synthesized. When it is completed, all of the chromosomes have been replicated, i.e., each chromosome has two (sister) chromatids. Therefore, during this phase, the amount of DNA in the cell has effectively doubled. G_2 is a gap that allows the cell to prepare for transition between S and M phases. The relatively short and highly regulated M phase starts with chromosome segregation into two nuclei and ends with the
formation of two daughter cells. The sequence of events in the complex process of mitosis is divided into various phases known as: prophase, metaphase, anaphase and telophase. Finally, cytokinesis phase that immediately follows mitosis divides cytoplasmic components of the cell equally between the two cells.

Fig 9: Schematic illustration of the cell cycle phases. (http://www2.le.ac.uk/departments/genetics/vgec/diagrams/22-Cell-cycle)

1.16.1 Regulation of cell-cycle

Since uncontrolled cell proliferation is the underlying cause of cancer, the critical phases of cell cycle are highly regulated by organized molecular mechanisms, also to encompass detection and repair of genetic damage and prevention of uncontrolled cell division. At least two types of cell cycle control mechanisms are recognized: The first type control mechanism constitutes cyclins and cyclin-dependent kinases (Cdk). Cdk's are the members of highly regulated kinase family that associate with regulatory subunit cyclins to create an active heterodimer complex with unique substrate specificity. Cdk’s are constitutively expressed in cells, whereas, cyclins are synthesized at specific stages of the cell cycle, in response to various molecular signals. The genes encoding these molecules are conserved among all eukaryotes. A cascade of protein
phosphorylations fine-tunes the activity of Cdk-cyclin complexes, ensuring well-delineated transitions between cell cycle stages (Fig 10) (85).

The various types of Cdk-cyclin complexes that control cell cycle phases are as follows. D cyclins are the first one to be produced in response to extracellular mitotic signal and important for transition from G₀ to S phase. It binds with Cdk4/Cdk6 for phosphorylation and inactivation of tumor suppressors retinoblastoma (pRb) protein family for expression of E2F transcriptional factors. Cyclin A functions in the S phase entry and transition, cyclins B1 and B2 are essential for G₂ exit and mitosis. Cyclin E binds to cdk2 and facilitates the cell from G₁ to S phase transition. It pushes the cell towards S phase. Cyclins B1 and B2 and cdk1 are M phase regulators. Cyclin B must be degraded for the cell to exit mitosis, thus accumulation of cyclin B delays the mitotic exit (86). The Cdk activity can be negatively regulated by Cdk inhibitors (CKI) such as members of INK4 family, by binding to the Cdk4 and Cdk6 kinases and preventing their interaction with D-cyclins, whereas, members of the Cip/Kip family (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}) form heterotrimeric complexes to inhibit the kinase activity of Cdk2/E-cyclins (87). Tumor suppressor protein 53 (p53) is an important regulator of cell cycle progression. It is activated upon cell stress or damage and is important for cell cycle arrest and induction of cell death (88). It induces transcription of the cyclin dependent kinase inhibitor-1 (p21) and thereby, inhibits proliferation of abnormal cells in the G₁ phase (89). The p53 can also inhibit the progression from G₂ to M, since activated TP53 can stimulate expression of the protein 14-3-3σ, which sequesters cyclin B/CDK in the cytoplasm, thus, arresting the cells in G₂ (90).

**Fig 10:** Regulation of cell cycle. (Nature reviews; molecular cell biology)
The second type of cell cycle regulation involves a set of checkpoints to monitor completion of critical events such as DNA replication and chromosome segregation and delay progression to the next stage, if necessary. Checkpoints typically consist of a network of regulatory proteins to ensure that damaged or incomplete DNA is not passed on to daughter cells. The three main checkpoints include: the G1/S checkpoint, the G2/M checkpoint and the metaphase (mitotic) checkpoint. While it is not essential for all the cells to pass through each of these checkpoints; as most of the cancers are developed due to mutations that allow the cells to rapidly pass through the various checkpoints or even skip them altogether.

1.17 Tumor-suppressor protein p53

The tumor-suppressor protein p53 is encoded by TP53 gene located on short arm of chromosome 17p13.1. It is known as ‘the guardian of the genome’ or ‘the cellular gatekeeper of growth and division’ (91). It acts as a key regulator of cellular growth control and plays a central role in the induction of genes that are important in cell cycle arrest and apoptosis following DNA damage. Under normal conditions, p53 levels are kept at low by its negative regulator mdm2 (mouse double minute 2), which facilitates ubiquitin mediated proteasomal degradation of the protein. p53 is a stress response protein that functions primarily as a tetrameric transcription factor, which regulates a large number of genes in response to a variety of cellular insults, including oncogene activation and DNA damage. These signals activate p53 primarily through posttranslational modifications that result in augmented p53 protein level and transactivation activity. Activated p53 suppresses cellular transformation mainly by inducing growth arrest, DNA repair and apoptosis in damaged cells (Fig 11) (92).

Fig 11: Schematic illustration of TP53 pathway following DNA damage (http://p53.free.fr/p53_info/image_info/G1_arrest)
p53 somatic mutations are one of the most frequent genetic alterations observed in human cancers, with significant variation in its incidence between cancer types, whereas, germ line mutations to the p53 gene cause a rare type of cancer predisposing condition known as Li–Fraumeni Syndrome(93). About 50% of CRCs are associated with p53 point mutations and vast majority (80%) of these are missense mutations comprising GC to AT transitions at CpG dinucleotides and occurs predominantly in five hotspot codons such as 175, 245, 248, 273 and 282 (94). Most of these mutations occur in a region, which contain the nucleotide sequences preserved during evolution and coding for the amino acids, which are extremely important for the p53 DNA binding activity (95). These mutations, usually lead to the formation of a full-length mutant protein, which is unable to activate target genes and prevent tumorigenesis. Moreover, these mutations, usually confer the mutant protein with a dominant negative activity over the remaining wild type allele, a mechanism that involves hetero oligomerization of the mutant p53 with the wild type protein (96) and these mutants also gain new oncogenic properties that are independent of wild type p53 known as the ‘gain of function properties’ (97).

The prognostic and predictive significance of p53 mutational status in CRCs may vary depending on the type of mutation (98) and the ethnic group, tumor site, stage of disease and use of adjuvant therapy (99, 100). However, evaluation of p53 over expression could find clinical application to identify CRC patients, who are likely to obtain benefit from the standard adjuvant chemotherapy. Therefore, it is critically important that the prognostic value of p53 is examined separately for patients treated with or without chemotherapy (101).

1.17.1 Apoptosis

Apoptosis or programmed cell death is an essential physiological process to control many aspects such as embryonic development, homeostasis, aging and immunity. It is characterized series of morphological changes such as, cell shrinkage, membrane blebbing, condensation of nuclear chromatin, cleavage of chromosomal DNA at internucleosomal sites and fragmentation of nucleus. Phosphatidylserine (located on the inner side of plasma membrane) becomes exposed on the outer surface, where it provides a recognition signal for uptake by phagocytes (102). Another specific feature of apoptosis is activation of a group of enzymes belonging to the cysteine-aspartic acid protease family namely caspases leading to cleavage of nuclear and
Caspases are produced as inactive monomeric proenzymes that undergo dimerization and often proteolytic cleavage at conserved aspartic residues for activation. They play central role by initiation and execution of apoptosis. Based on their role in apoptosis, they are classified as initiator caspases (8, 9) and executioner caspases (3, 6, 7). There are two pathways by which it is activated such as extrinsic (death receptor) and intrinsic (mitochondria) pathways of apoptosis. These pathways, however, are not exclusive, and evidence suggest that they can be linked and influence each other.

Extrinsic pathway is initiated by binding of death ligands to death receptors. Though there are several receptors, the best known death receptors are TNFR1 and FAS receptors and their ligands are called TNF and FAS ligands respectively. Binding of ligands to their receptor activates pro-caspase-8 which is an initiator caspase of apoptosis resulting cleavage and activation of executioner caspases.

On the other hand, intrinsic pathway is initiated due to stimuli such as genetic damage, hypoxia and severe oxidative stress. This pathway is closely regulated by a group of proteins belonging to the Bcl-2 (B-cell lymphoma) family. There are two main groups of apoptotic proteins such as pro-apoptotic proteins (e.g., Bax, Bak, Bad, Bcl-Xs, Bid, Bik and Bim) and the anti-apoptotic proteins (e.g., Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1). The release of cytochrome c into the cytoplasm due to increased mitochondrial permeability activates caspase-3. The anti-apoptotic proteins regulate apoptosis by blocking the mitochondrial release of cytochrome c, whereas, the pro-apoptotic proteins promote its release. The balance between pro- and anti-apoptotic proteins determine whether, apoptosis is initiated or not. The execution phase of apoptosis involves the activation of series of caspases. The upstream caspase for intrinsic pathway is caspase-9 and for extrinsic pathway is caspase-8. Both of these pathways converge to caspase-3, which, in turn, cleaves the inhibitor of caspase activated deoxyribonuclease resulting in the nuclear fragmentation. Other downstream caspases induce cleavage of cytoskeletal proteins, DNA repair proteins, protein kinases, together contributing to the typical morphological changes in apoptosis (104).

Deregulation of apoptosis contributes to multiple diseases including cancer. Diminished activity of apoptosis is clearly implicated as one of the causes of cancer development by failure to
eliminate potentially malignant cells, while an enhanced activity is also clearly linked to many pathological conditions, including neurodegenerative and autoimmune diseases. There are many ways a malignant cell can evade apoptosis or acquire resistance. The mechanisms by which evasion of apoptosis occurs are broadly classified into 1) improper balance of pro- and anti-apoptotic proteins; 2) diminished caspase activity and 3) impaired death receptor signaling. On the other hand, explicit knowledge on apoptosis offers to identify novel therapeutic targets. Every defect or deregulation in apoptosis pathway may be exploited as an attractive target of cancer treatment. Many of such apoptosis based therapeutic strategies are under development such as agents that target Bcl-2 family proteins; p53 based gene/drug therapy; caspase based therapy and inhibitor of apoptosis proteins antagonists (105).

1.18 Rationale of the current study

Currently used chemotherapeutic regimens employ a combination of anticancer agents that target different cellular pathways to obtain high clinical efficacy has become a regular practice in treating cancer. Limonene has been selected to test in CRC cells because, natural dietary compounds with anticancer activity have been shown to provide significant protection against CRC because of their proximity during digestion. BEZ was selected based on the observation that drugs targeting both class IA PI3Ks and the catalytic site of mTOR are more effective and less toxic than using two different agents to target same pathway. While both, limonene and BEZ-235 have been individually shown to be effective in various cancers in preclinical studies, the combination of these drugs has not been tested before in CRC. Aberrant activation of PI3K/AKT pathway may lead to chemoresistance and thus, cancer cells become less sensitive to drug induced cell death. Several lines of experimental evidence also indicate that drugs targeting this pathway might be more effective (77). Although BEZ-235 is a dual inhibitor of PI3K/mTOR, when used for prolonged periods at lower concentrations, caused sustained inhibition of mTORC1 and mTORC2, whereas, the inhibition of PI3K activity was only transient (54). As a result, the cells may become less sensitive to apoptosis and the clinical efficacy of the drug might be decreased. On the other hand, limonene has been shown to inhibit PI3K/AKT activity in cancer cells. The chemotherapeutic potential of limonene had been demonstrated in several preclinical and clinical studies and in animal models of chemically induced tumors, however, the anticancer effects of limonene in CRC have not been investigated in detail earlier.
So, it is our hypothesis that a novel combination of limonene and BEZ-235 will likely to yield enhanced efficacy to the tune of synergistic anticancer effects; possibly by imposing a sustained inhibition on PI3K/AKT pathway, therefore, sensitizing the cancer cells towards death. The proposed hypothesis is planned to investigate systematically in a panel of CRC cells described below.

1.19 Choice of colorectal cancer cells for the study

The value of these tumor cell lines, as research models is greatly enhanced when there is an understanding of the underlying genetic abnormalities that drive their phenotype. Human CRC cell lines such as COLO-320, HCT-116, HT-29 and SW-620 cells have been proposed to use in the present study.

1.19.1 COLO-320 human colorectal cancer cells

These cells were established from the tumor mass of a 55-year-old caucasian woman with a moderately undifferentiated adenocarcinoma of the sigmoid colon in 1977 (106). COLO-320 cells exhibit mixed culture properties as they appear round cells in suspension and loosely adherent in monolayers. The cell line is negative for carcinoembryonic antigen and produces several hormones such as serotonin, norepinephrine, epinephrine, adrenocorticotropic hormone and parathyroid hormone. These cells express wild-type K-ras and PIK3CA genes and mutant p53 at the hotspot codon 248 (R248W). This cell line is tumorigenic in nude mice and one of the most commonly used cell line for *in vitro* studies.

1.19.2 HCT-116 human colorectal cancer cells

HCT-116 cell line is of epithelial origin deposited by MG Brattain from a male colorectal carcinoma patient. This cell line is positive for K-ras mutation in codon 13 (G13D) which is recognized as one of the hot-spots for K-ras mutations. The G13D mutation results in an amino acid substitution at position 13 in K-ras, from a glycine (G) to an aspartic acid (D). The frequency of G13D mutations among K-ras mutated CRC is 18.9–19.2% (107). The cell line is also positive for PIK3CA mutations (H1047R) that occurs predominantly within a highly conserved kinase domain in exon 20 promoting constitutive signaling. This mutation results in an amino acid
substitution at position 1047 in PIK3CA from histidine (H) to arginine (R). HCT-116 cells express wild type p53. It is tumorigenic in nude mice and one of the most commonly used cell line for in vitro and in vivo xenograft studies.

1.19.3 HT-29 human colorectal cancer cells

The human colon adenocarcinoma cell line HT29 was isolated from a primary tumor of a 44 years old caucasian female in 1964 by Fogh and Trempe (108). These cells contain wild type K-ras and mutant PIK3CA (P449T). The cell line also contains mutant p53 at codon 273 (R273H) which is considered as one of the hotspots for p53 mutations in CRC and occurs in the DNA binding domain of the protein (109). Because of their ability to express different pathways of enterocyte differentiation under standard culture conditions, HT-29 cells have become a unique model for studying the structural and molecular mechanisms involved in intestinal cell differentiation (110). The various soluble factors secreted from cells in culture medium include metabolites, cytokines, growth factors, etc., are known to promote cell survival. It was suggested, based on previous reports on biopsy samples, that a similar cytokine secretion profile can be observed in vivo also (111). It is also one of the cell line showing greater stability, consistency and reproducibility in its differentiation characteristics even after high passages (112). Over all, the scientific importance of HT-29 cell line is not only limited to study the biology of human CRCs, because of its ability to express features of enterocyte, it is indeed the most preferred cell line for studies focused on food digestion and bioavailability, epithelial response to microbial infections such as survival, adhesion or invasion (113), role of mucin secretion (114), probiotic adhesion and delivery systems.

1.19.4 SW-620 human colorectal cancer cells

The human colorectal adenocarcinoma cell line SW620 was derived from the lymph node of a 51-year-old caucasian male by A. Leibovitz, et al (115). The cell line was derived from a metastasis of the same tumor from which the SW480 was also derived. Both of these cell lines have been used for a number of biochemical, immunological, and genetic studies on CRC (116). SW-620 cell line contains mutant K-ras (G12V) and wild type PIK3CA. The G12V mutation results in an amino acid substitution at position 12 in K-ras from glycine (G) to valine (V). There
is a G -> A mutation in codon 273 of the p53 gene resulting in an Arg -> His substitution. The established cell line consists mainly of individual small spherical and bipolar cells lacking microvilli and synthesizes only small quantities of carcinoembryonic antigen, and highly tumorigenic in nude mice. This cell line is also suitable for transfection studies. The base medium for this cell line is ATCC-formulated Leibovitz's L-15 Medium, for use in a free gas exchange with atmospheric air.

**Table 2:** Colon cancer cells and their mutational status in reference to K-ras and PI3K pathway

<table>
<thead>
<tr>
<th>Cell line</th>
<th>K-ras</th>
<th>PI3K</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLO-320</td>
<td>Wild</td>
<td>Wild</td>
</tr>
<tr>
<td>HCT-116</td>
<td>Mutation</td>
<td>Mutation</td>
</tr>
<tr>
<td>HT-29</td>
<td>Wild</td>
<td>Mutation</td>
</tr>
<tr>
<td>SW-620</td>
<td>Mutation</td>
<td>Wild</td>
</tr>
</tbody>
</table>

Human CRC cells COLO-320 (wt-K-ras and PI3K), HCT-116 (mt-K-ras and PI3K), HT-29 (wt-K-ras and mt-PI3K) and SW-620 (mt-K-ras and wt-PI3K) have been chosen to test the hypothesis. It is also known that mutations either in K-ras or PI3K are used as biomarkers while designing chemotherapy regimens and also in the clinical assessment of CRC. These CRC cells were proved as valuable tools for anticancer drug screening because of their scope to cover-up wide molecular types of CRCs. Moreover, recent studies also showed that both K-ras and PI3K mutations coexists in a number of human CRCs leading to poor prognosis and CRC cells having coexisting mutations displayed additive signaling of PI3K/AKT as indicated by elevated levels of phosphorylated AKT and mTOR leading to inhibit apoptosis. Therefore, the results of the present study (*in vitro*) could be directly correlated to assess the clinical utility of the drugs/combination in CRCs of varied phenotypes and also useful in planning clinical studies (*in vivo*) later.

In addition, CRCs are considered as the most resistant type because of their constant exposure to ingested food and toxins and thus CRC cells are considered as an ideal model for representing clinical cancer drug resistance. Therefore, testing the drugs/combination in selected CRC cells
may reveal the clinical efficacy of the drug combination against even most resistant cancer phenotypes by exceeding drug resistance.

1.20 Aims and Objectives

The anticancer activity of limonene and BEZ combination in CRC cells is proposed to investigate using a battery of in vitro anticancer tests. These assays in general, provide mechanistic insights in understanding the anticancer activities of the drug combination and are suitably validated, therefore, the results obtained here are considered as valid. Moreover, in vitro findings form the background based on which further more complex in vivo experiments or clinical testing can be planned. Although there are vast number of anticancer tests, based on the proposed hypothesis and existing knowledge on test compounds, the testing of limonene and BEZ combination was limited to the assays listed below, covering the fundamental yet the key aspects of anticancer activity such as antiproliferative activity, antitumor and antimetastatic activity, regulation of cell cycle phases, induction of apoptosis and regulation of phosphorylation of effectors of key cell signaling pathways (PI3K/mTOR) in cancer cells.

- Testing the combination efficacy of limonene and BEZ anticancerous activity studying the parameters of cell viability/anti-proliferative activity in the following CRC cell lines:
  - COLO-320
  - HCT-116
  - HT-29 and
  - SW-620
- Testing the combination effects on cancer cells for:
  - Antitumor activity by performing clonogenic ability (colony formation assay) and
  - Antimetastatic activity by in vitro scratch assay for cell migration
- CompuSyn analysis of combination effects on cell viability, colony formation assay and cell migration to determine synergy/additive/antagonistic interactions
- Studying the effects of drugs on cell cycle distribution using flow cytometry analysis
- Determining the combination effects on apoptosis by evaluation of:
  - Fluorescent microscopic examination of apoptotic cells-DAPI staining
• Estimation of caspase 3- by colorimetric assay
• Estimation of caspase 9- by colorimetric assay
• Pro-apoptotic proteins- BAD, BAX- Western blot analysis and
• Anti-apoptotic proteins- Bcl-2- Western blot analysis

❖ Effect of combination treatment on p53 activity by Western blot analysis of CRC cells containing wild and mutant p53:
  • HCT-116 wt-p53 and
  • HT-29 mt-p53(R273H)

❖ Effects on PI3K/mTOR pathway- by Western blot analysis to estimate key phosphorylated proteins of the pathway
  • PI3K (p-AKT\textsuperscript{Thr308})
  • mTOR 1(p-70S6K\textsuperscript{Thr389}) and
  • mTOR 2 (p-AKT\textsuperscript{Ser473}).