CHAPTER-1

Introduction

Carcinogenesis is a multistep process typically occurring over an extended period as it takes many years to turn into complete malignancy, and comprises three major steps that reflect genetic alterations: initiation (normal cell transformed or initiated cell), promotion (initiated cell →preneoplasic cell) and progression (preneoplasic cell →neoplasic cell). These concepts although represent a simplification of the real process, they are very useful to understand the natural history of cancer. Initiation process is moderately short and needs a chronic exposure to endogenous or exogenous genotoxic agents (chemical products, physical radiations or biological agents) that induces sporadic or inherited mutations. The tumor promotion stage is a long term and irreversible process in which transformed cells can increase the genetic damage produced during the initiation and proliferate under the action of different stimuli (hormones, growth factors, some dietary lipids, etc.), influencing the later growth and clonal expansion of abnormal cells (Brennan, 1975; Nowell, 2002; Thangapazham *et al.,* 2006). Finally, the third phase occurs when the cell undergoes additional genetic alterations that lead to the expression of the malignant phenotype. During this phase, the cells show a marked genomic instability and acquire the ability to infiltrate adjacent tissues and metastasizing power (Nowell, 2002).

Colorectal cancer (CRC) is one of the most common gastrointestinal tract malignancies. It is the fourth most common form of cancer worldwide and affects about one million people every year throughout the world with a high mortality rate. In the United States, CRC ranks second among the cancer related deaths. It occurs with equal frequency in both men and women. The number of new cases of CRC has been rapidly increasing since 1975. It is estimated that nearly 150,000 new cases of CRC are diagnosed in the United States each year (Jemal *et*

al., 2005). The increased incidence of CRC in the western world has partly been attributed to dietary factors such as a high-fat and low fiber-diet (Negri *et al.,* 1998). CRC develops from a dysplasic precursor lesion, sporadically, in the context of high-risk hereditary conditions, or in the background of chronic inflammation.

Generally CRC arises as a result of sequential episodes of activating mutations in oncogenes, such as *k-ras*, and inactivating mutations, truncations or deletions in the coding sequence of several tumor suppressor genes, including p53 and adenomatous polyposis coli (APC), together with aberrant activity of molecules controlling genomic stability (Vogelstein & Kinzler, 2004). In particular, there are two main genomic instability that contribute to colon carcinogenesis: chromosomal instability (CIN) and microsatellity instability (MSI). The first results in abnormal segregation of chromosomes and abnormal DNA content (aneuploidy). The second involves loss of function of oral genes that normally repair the mismatches between DNA base pairs that occur during the normal process of DNA replication in dividing cells (Itzkowitz, 2006).

Three genetic patterns have been categorized into the following groups of CRC: sporadic, inherited and familiar CRC. In hereditary cancer, such as familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) some of these genetic mutations are inherited, while in sporadic cancer the mutations occur spontaneously. Particularly, the loss of a functional APC protein is one of the earliest events occurring in sporadic CRC suggesting that APC may act as a gatekeeper of the colonic epithelium (Vogelstein & Kinzler, 2004). Loss of APC function allows β-catenin, a protein which plays a role in both cell adhesion and intracellular signaling, to gain access to the cell nucleus, where it complexes with specific transcription factors to activate genes implicated in proliferation, apoptosis and cell adhesion. APC also plays a role in epithelial migration and can localize at kinetochores, thereby participating in chromosome segregation during mitosis, which may contribute to the genetic instability and loss of epithelial polarity during the malignant transformation of colonic epithelia (Castellone *et al.,* 2006).

In addition to hereditable factors, cancer risk appears to be markedly influenced by a number of different factors including chronic inflammation. In effect, patients with inflammatory bowel disease (IBD) are among the highest risk groups for developing CRC. Increased risk of CRC in these patients becomes greater with increasing extent and duration of the disease (Itzkowitz, 2006). At present no genetic basis is able to explain the predisposition to CRC in those patients. Nevertheless, the main genomic instability that contributes to colon carcinogenesis in addition to MSI instability is CIN which results in damage of genetic material and consequently, loss of function of key tumor suppressor genes such as APC and p53 which express proteins that regulate growth and apoptosis. Loss of APC function occurs later normally in colitis-associated CRC. In normal cells, p53 is inactive through its joining to the MDM-2 protein, but several stimuli can activate it, resulting in anticancer responses: activation of genes involved in inhibition of cell cycle, apoptosis, chromosomic stability and inhibition of angiogenesis. Loss of p53 gene function occurs early and is supposed to be the crucial event that drives the adenoma to carcinoma (Itzkowitz, 2006; Burmer *et al.,* 1992).

CRC is diagnosed either after routine screening or prompted by the onset of new symptoms. Symptoms in CRC are nonspecific and vague, and may include a change in bowel habits, weight loss, abdominal pain, and fatigue. Rarely, more specific symptoms such as obstruction, bleeding, or perforation may occur, prompting an urgent surgery (Labianca *et al.,* 2010). The goal of preoperative imaging is to accurately stage patients. This usually entails, at minimum, a CT scan of the abdomen and pelvis. Chest imaging to evaluate for pulmonary metastases may consist of either plain films or CT scan, depending on physician preference and regional standards (Weitz *et al.,* 2005). Positron emission tomography (PET) has excellent sensitivity and specificity in detecting metastatic disease in CRC, but has limited sensitivity for lymph node involvement, although significantly higher than CT (Kosugi *et al.,* 2008; Shin *et al.,* 2008) The combined PET/CT is an emerging test that combines into one study the anatomical data from the standard CT scan with the functional data

obtained from PET. It has been suggested that PET/CT has improved sensitivity for identification of extra hepatic metastases (Park *et al.,* 2006; Selzner *et al.,* 2004). However, there is currently no algorithm for preoperative imaging evaluation of CRC.

Traditionally, the mainstay of chemotherapeutic treatment has been intravenous (IV) 5-fluorouracil (5-FU) in conjunction with leucovorin (LV). LV, also known as folinic acid, is known to improve tumor response rates (RRs) when combined with 5-FU, translating into longer disease-free survival (DFS) and overall survival (OS) (Thirion *et al.,* 2004; Moertel *et al.,* 1990). Starting in 2000, several new and exciting agents have been introduced into the battle against metastatic CRC. Capecitabine, an oral derivative of 5-FU, was introduced in 2005 after displaying equivalent efficacy compared to IV 5-FU (Hoff *et al.,* 2001). Irinotecan, an inhibitor of topoisomerase I, was introduced in 1996 and is currently approved as both primary therapy (i.e., FOLFIRI [LV/5-FU/irinotecan]) and monotherapy after 5-FU failure (Douillard *et al.,* 2000; Köhne *et al.,* 2005; Cunningham & Starling, 2007). Investigators have begun evaluating the impact of substituting oral capecitabine for IV 5-FU (XELIRI [capecitabine/irinotecan] regimen). This regimen remains in phase II trials and has yet to be compared head to head with FOLFIRI (Cremolini *et al.,* 2015).

Oxaliplatin, a platinum derivative, was introduced in 2002. Oxaliplatin is not indicated for monotherapy, but has dramatically improved progression-free survival (PFS) and OS in combination with the FOLFOX (LV/5-FU/oxaliplatin) regimen, which is currently first-line treatment in the United States for advanced and metastatic CRC (Kuebler *et al.,* 2007; André *et al.,* 2009). In comparison to 5-FU/leucovorin, patients with stage IV disease treated with FOLFOX showed a significantly longer PFS (9 vs 6 mo, P =0.001) and superior RR (50% vs 22%, P = 0.001) (De Gramont *et al.,* 2000). All chemotherapeutic agents have associated toxicity, notably oxaliplatin, has a high incidence of sensory neuropathy that usually occurs after a cumulative dose of 640 mg/m^2 (McLeod *et al.,* 2010). The assessment of XELOX (capecitabine/oxaliplatin) versus FOLFOX has shown no significant difference between the 2 arms and concludes that XELOX may be

indicated as first-line treatment in appropriate patients (Cassidy *et al.,* 2008). Two large multicenter trials found no significant difference in PFS or OS between the 2 regimens (Tournigand *et al.,* 2004). The main disparity was the toxicity profile, with FOLFOX affecting the nervous system and FOLFIRI affecting the gastrointestinal system (Colucci *et al.,* 2005). Therefore, to avoid these side effects, there is an urgent need to discover more potent anticancer agents with no side effects on normal organs/tissues.

Recent studies have shown that curcumin (CUR) and naringenin (NAR), one of the most promising natural chemotherapeutic agents, are used extensively to treat a variety of solid tumors, including stomach, breast, intestine and especially colorectal carcinoma (Aggarwal *et al.,* 2003). CUR and NAR are potentially involved in the reduction of carcinogenesis by protecting DNA damage (induction), inhibiting tumor growth (progression) and regressing tumor invasion (proliferation) (Tripoli *et al.,* 2007). CUR (a turmeric derivative), a member of the ginger family Zingeberacea, has been extracted from the dried ground rhizome of the perennial herb curcuma species (*Curcuma longa*). It is a yellow color polyphenolic natural compound used as a spice to give the specific flavour and colour to curry (Calabrese *et al.,* 2003; Ammon & Wahl, 1991), whereas NAR is one of the naturally occurring flavonoids present in citrus fruits, cherries, grapefruits, tomato and cocoa, with no discernible toxicity. NAR possesses three hydroxyl substitutents at C-4', C-5 and C-7 position of aromatic ring resulted in better inhibitory effects on CRC and has widespread pharmacological property (Van Acker *et al.,* 2000). In spite of vast practical applications and therapeutic effectiveness of CUR and NAR in a variety of experimental models, its clinical applications have been hampered due to its extreme water insolubility and instability in physiological medium (Ammon & Wahl, 1991; Manthey *et al.,* 2001). Low aqueous solubility of CUR and NAR results in poor bioavailability, poor permeability and extensive first pass metabolism before reaching into the systemic circulation (Anand *et al.,* 2007; Hsiu *et al.,* 2007). To overcome these problems, nano-drug delivery system is

expected to yield more promising clinical applications by delivering both CUR as well as NAR.

Present thesis embodies to design and optimize: first lipopolysaccharide based oral nanocarriers and second polymeric nanoparticles of CUR and NAR for the enhancement of their bioavailability and anticancer efficacy in CRC. Important features of nanocarriers include their biocompatibility, high drug loading capacity, possibility of loading both hydrophobic and hydrophilic compounds and favorable pharmacokinetic profile (De Jong & Borm, 2008). When administered through oral route, they get internalized by M-cells of Payer's patches in gut lumen and subsequently transported through lymphatic system into the blood stream and thus, enhance the bioavailability of encapsulated drug. Further, the leaky vasculature in solid tumors help in delivering nanocarriers to the tumor site through the enhanced permeation and retention (EPR) mechanism and also, reduces P-gp-mediated multidrug resistance in cancer cells thereby, increases efficacy of anticancer agents (Mei *et al.,* 2013). Additional benefits offered by nanocarriers are easy to scale up, avoid use of organic solvents during preparation, stability against self-aggregation and degradation due to encapsulation of drug inside nanocarriers make the system superior over other systems (Mehnert and Mader, 2001). Polymeric nanoparticles can improve the therapeutic activity and safety of drugs, mainly by delivering them to their site of action and by maintaining therapeutic drug levels for prolonged periods (Ratnam *et al.,* 2006). Due to their small size, this system can offer many advantages which include protection of drugs from enzymatic and gastric degradation in the gastrointestinal tract, enhancement of bioactivity and bioavailability, sustaining the effect in the target tissue, cellular/tissue targeting and improved efficacy (Barzegar-Jalali *et al.,* 2012). Further, it is well known that polymeric nanoparticles can reach tumor tissues passively through the leaky vasculature surrounding the solid tumors by a same process called EPR mechanism (Acharya & Sahoo, 2001). Therefore, nanocarriers system and polymeric nanoparticles could act as an efficient delivery system for improving both bioavailability as well as anticancer efficacy of CUR and NAR, respectively.

The process of formulation development and their optimization involves a vital understanding of the effect the formulation factors viz. drug: polymer ratio, concentration of surfactants, volume of organic solvent, homogenization speed etc. on the properties of the formulation (response) such as mean particle size, percent entrapment efficiency, etc. Conventional optimization technique involves altering one independent factor at a time and keeping all others constant, which only allows to assess the impact of one particular parameter on the process performance. This approach is time consuming, requires large number of experiments, costing high and also cannot provide information regarding the mutual interactions of parameters. To defeat the limitations of conventional approach, one of the widely used industry based systematic approach Quality by Design i.e. Taguchi orthogonal experimental design was employed. This approach is a powerful problem solving tool for improving the process performance, yields and productivity without compromising quality of the product. A large number of process variables with different parameters can be studied with Taguchi robust design by employing orthogonal arrays based minimum number of experiments (Al-Refaie & Al-Tahat, 2011). Three major tools used in the Taguchi method are the orthogonal arrays, analysis of variance (ANOVA), and the mean/signal-to-noise ratio (S/N). In the application of robust design, Taguchi method defines two types of variables or factors in the system: control factors and noise factors. A control factor can be selected and fixed at a certain level. On the contrary, a noise factor cannot be controlled because of practical, economic, or other reasons (Taguchi, 1986). Taguchi method constitutes an effective tool for selecting the best combination of levels of control factors of a system to obtain a combination of factors that enable a robust behavior against the variation of noise factors (Mousavi *et al.,* 2007). A robust solution is the combination of factors whose variation does not produce a sensible change in the response. This methodology aims to reduce the undesirable effects caused by all noise sources present in the observed response of the system. For this reason, it is necessary to determine whether a difference exists between the magnitudes of level errors of a control factor. If there is a significant difference in magnitude, we could select the level of control factor

with the smallest error, thus determining the most robust condition. The selection of more robust levels is accomplished by calculating and comparing the S/R or S/N. This parameter is therefore the best index to measure quality in a robust model (Madu & Madu, 1999).

After evaluating the main and interaction variables which affect properties of formulations viz. mean particle size and percentage entrapment efficiency, a four-factor three-level (3^4) was employed to schedule and perform the experiments. Soluthin MD® and Eudragit® E 100 was used as drug carrier polymers. Soluthin MD® is an approved new class of lipopolysaccharide based hydrophilic compound, composed of major carbohydrate component as 80% maltodextrin and minor lipid component as 20% phosphatidylcholine, which act as solubilizer, bioavailability enhancer, biodegradable and biocompatible carrier (Chen & Chen, 2012). Whereas, Eudragit® E 100 cationic copolymer consisting of 1:2:1 ratio of methyl methacrylate, N,N-dimethylaminoethyl methacrylate and butyl methacrylate monomers, has been widely employed to improve the solubility of poorly water-soluble drugs (Jung *et al.,* 1999). The lipopolysaccharide based nanocarriers and polymeric nanoparticles were prepared by high speed homogenization technique as well as emulsification-diffusion-evaporation method, respectively (Noack *et al.,* 2001; Fessi *et al.,* 1989). The optimized formulations of CUR and NAR were developed based on predicted optimum levels of the independent variables of the Taguchi orthogonal arrays and evaluated for their *in-vitro* as well as *in- vivo* models. The optimized formulations were evaluated for various physicochemical characteristics such as mean particle size, percentage entrapment efficiency, percentage drug loading, polydispersity index and zeta potential whereas solid state characterizations was done by fourier transform infrared spectrophotometer, differential scanning calorimetry and powder X-ray diffraction studies. The surface morphology of the optimized formulations was visualized using high resolution transmission electron microscopy and atomic force microscopy. The drug distribution within the nanocarriers was examined by using confocal laser scanning microscope. The photophysical properties of drug were further examined by UV-Visible

spectroscopic analysis. Further, the optimized formulations were evaluated for *in-vitro* drug release study in PBS, pH 7.4 at the end of 24 hr, stability study at room temperature (25±2°C) and standard relative humidity (60±5% RH) for 3 months, *in-vivo* pharmacokinetic studies using Wister rats and *in-vitro* cytotoxicity study on colon-26 cancer cell line. In addition, the *in-vivo* anticancer efficacy of optimized formulations was also evaluated in murine colon-26 tumor-bearing BALB/c mice. Furthermore, the safety of the optimized formulations was assessed by observing changes in the body weights and survival rates of the tumor-bearing mice.