CHAPTER 1
INTRODUCTION

1.1 Colon cancer

Colorectal cancer is defined as the cancer affecting the cecum, ascending, transverse, descending, sigmoid colon and finally the rectum. The types of cancers in the colon and rectum consist of adeno carcinomas, carcinoid tumors, gastrointestinal stromal tumors, lymphomas and sarcomas (1). It starts when the process of the normal replacement of lining cells is lost, which results in abnormal mucosal cell division. These abnormal cells grow and divide and lead to the formation of precancerous tumors known as polyps. As polyps grow, additional genetic mutations further destabilize the cells and can make the cells malignant. Once a colorectal cancer forms, it will grow locally and extend further through the wall of the intestine and invade adjacent structures making the primary tumor and it is harder to remove. In addition, local extensions can lead to additional symptoms such as pain, fullness, and causes blockages of the colon or nearby structures. Further the cancer begins to metastasized, to distant locations (2-5). The major risk factors for colorectal cancer include,

- High fat intake
- A family history of colorectal cancer and polyps
- Presence of polyps in the large intestine
- Inflammatory bowel diseases, primarily chronic ulcerative colitis

1.1.1 Colon cancer statistics

Colon cancer is one among the 40% of all cancer cases diagnosed yearly and this accounts for the third most leading cause of cancer death in males and fourth
most in females in the U.S. This year’s report estimates that there will be 1,665,540 new cancer cases and 585,720 cancer deaths in the United States in 2014. Among men, cancer of the colon along with prostate and lung will account for about half of all newly diagnosed cancers, with prostate cancer alone accounting for about one in four cases. Among women, the three most common cancers in 2014 will be breast, lung, and colon, which together will account for half of all cases (4-6). In India, the colorectal cancer incidence rate is 45.2 and 38.0 respectively for males and females. The mortality rate includes 18.8 and 14.6 respectively for Indian males and female populations respectively (1).

1.1.2 Colon cancer diagnosis and treatment

The diagnosis of colon cancer can be made by fecal occult blood test (FOBT), fecal immunochemical test (FIT), stool DNA test, barium enema or by sigmoidoscopy and colonoscopy with biopsy confirmation of cancer tissue. The treatment of colorectal cancer depends on the location, size, and extent of cancer spreading, as well as the health of the patient (7, 8). The treatment modalities for colon cancer include surgery, radiofrequency ablation, cryosurgery, chemotherapy, radiation therapy and targeted therapy. Among which chemotherapy utilizes different drugs or drug combination to reduce the cancer cell growth. In colon cancer, chemotherapy is given adjuvantly after surgery and it become a major part in colon cancer treatment (9).

1.1.3 Colon cancer treatment using chemo drugs and their limitations

The FDA approved chemo drugs or drug combinations used in colon cancer include adrucil (5-fluorouracil, 5-FU), avastin (bevacizumab), camptosar (irinotecan hydrochloride), capecitabine, cetuximab, eloxatin (oxaliplatin), erbitux (cetuximab) or drug combinations such as capox, folfiri, folfiri-bevacizumab, folfiri-cetuximab, folfox, zelox etc (9). In colon cancer, chemotherapy is given adjuvantly after surgery and the first line chemo drug used is 5-FU (9, 10). 5-FU; being a thymidylate synthase inhibitor, it inhibits the cancer cell growth through the arrest of cells in the S phase (11). 5-FU usage in chemo treatment is restricted due to its major drawback such as systemic toxicity arises from its non-specificity, low plasma half life leading to the use
of high doses (12), its inefficacy in chemo treatment results from cyclooxygenase 2 (COX-2) over expression in colon cancer (13-17) and 5-FU resistance(18-20).

1.2. Review of literature

1.2.1 5-FU as the first line chemo drug against colon cancer

5-FU trademarked as Adrucil (IV), Carac (topical), Efudex (topical)) is a drug that is a pyrimidine analog, belongs to the family of anti-metabolites, which is used in the treatment of cancer. Medical uses of 5-FU include systemic administration applications for anal, breast, colorectal, oesophageal, stomach, pancreatic, skin and head and neck cancers (21). It has also been given topically for actinic keratoses and Bowen's disease (21). The credit of design, and synthesis of 5-FU goes to Charles Heidelberger and it has been patented during the year 1957 (22). Fig.1.1 Depicts the chemical structure of 5-FU (23). 5-FU has been used as the first-choice chemotherapy drug for colon cancer for many decades (24, 25) and it has been approved by the food and drug administration (FDA) since 1991(26).

![Figure 1.1. Depicts the chemical structure of 5-FU (23).](image)

1.2. 2 Mechanism of action of 5-FU

The chemotherapeutic agent 5-FU acts in several ways, but principally as a thymidylate synthase (TS) inhibitor. Interrupting the action of this enzyme blocks synthesis of the pyrimidine thymidine, which is a nucleoside required for DNA replication. TS methylates deoxyuridine monophosphate (dUMP) into thymidine monophosphate (dTMP). Administration of 5-FU causes a scarcity in dTMP, so rapidly dividing cancerous cells undergo cell death via thymine less death (11).
Calcium folinate provides an exogenous source of reduced folinates and hence stabilize the 5-FU-TS complex hence enhancing 5-FU’s cytotoxicity (11).

1.2. 3 Metabolism of 5-FU

5-FU is converted to three main active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The main mechanism of 5-FU activation is the conversion to fluorouridine monophosphate (FUMP), either directly by orotate phosphoribosyltransferase (OPRT) with phosphoribosyl pyrophosphate (PRPP) as the cofactor, or indirectly via fluorouridine (FUR) through the sequential action of uridine phosphorylase (UP) and uridine kinase (UK). FUMP is then phosphorylated to fluorouridine diphosphate (FUDP), which can be either further phosphorylated to the active metabolite fluorouridine triphosphate (FUTP), or converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RR). In turn, FdUDP can either be phosphorylated or dephosphorylated to generate the active metabolites; FdUTP and FdUMP, respectively. An alternative activation pathway involves the thymidine phosphorylase catalyzed conversion of 5-FU to fluorodeoxyuridine (FUDR), which is then phosphorylated by thymidine kinase (TK) to FdUMP. Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumor cells. Up to 80% of administered 5-FU is broken down by DPD in the liver (11).

1.2. 4 Important facts about the use of 5-FU as a chemo drug in colon cancer

• No drug with superior antitumor activity has been developed since the introduction of 5-FU into clinical practice 3 decades ago.

• Objective tumor responses are observed in only 15 to 20% of patients treated with 5-FU, and there has been no evidence that such treatment improves patient survival.

• The rate of colorectal tumour regression of patients with 5-FU chemotherapy ranged from 8 to 85% and there were no clear evidence for the improved survival of patients with this drug (27).
• Combination chemotherapeutic regimens have not been shown to improve survival, and the clinical value of any increase in tumor response rate has been mitigated by an increase in toxicity.

• Over all the chemotherapy with 5-FU alone is restricted due to the following reasons (5-FU’s efficacy is restricted due to the following reasons),
  ➢ Low plasma half life leading to the use of high doses within a short span, which finally results in unwanted toxic side effects to the healthy normal cells (28-30).
  ➢ Due to 5-FU drug resistance (31-33).

There are different drug combination studies carried out to improve the drug resistance of 5-FU in carcinoma of colon and hepatic cancers (HCC) and it is described in the Table 1.1.

<table>
<thead>
<tr>
<th>Drugs used in combination with 5-FU</th>
<th>Type of study</th>
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• Dose dependent ATO mediated thymidylate synthase (TS) inhibition leads to the sensitization of 5-FU to HepG2 cells. | (34) |
| Sorafenib                         | *In vitro* anticancer effects in hepatocarcinoma (HCC) cell lines. | • Individual as well as the combinatorial antitumor activity of sorafenib and 5-FU was seen in HCC cell lines. However the nature of the effect depends up on the particular cell line and treatment order of the two compounds. | (35) |
### ATO

| The chemo sensitizing effects of ATO towards 5-FU was evaluated in 5-FU sensitive and resistant colorectal cancer cells (HT29). | Combination treatment increased the cytotoxicity of both 5-FU sensitive and resistant cancer cells due to the specific inhibition of ATO towards TS and p53 gene expression. | (36) |

### Sorafenib

| To analyze the effect of sorafenib single treatment versus combination treatment in human colorectal cancers. | The treatment effects were partially cancelled when 5-FU and sorafenib were applied simultaneously. | (37) |

### Rosemary extract (SFRE) [Extraction through supercritical fluid technology].

| The anticancer effects of SFRE both alone and in combination with 5-FU were evaluated in sensitive and resistant human colon cancer cells. | SFRE showed synergistic anticancer effects in combination with 5-FU in colon cancer cells through the down regulation of TS and thymidine kinase enzyme (TK1), by SFRE. Since these TS and TK1 enzymes are responsible for 5-FU resistance in colon cancers. | (38) |

### Celecoxib (Cox-2 inhibitor)

| Combinatorial in vivo anti-tumor evaluation of 5-FU with celecoxib in human colorectal cancer model. | Significant reduction in tumor growth was observed in combination treatment. The anti-tumor effects were the result of the apoptosis induction through the activation of cytochrome c, caspase-3 and caspase-9 which are involved in the apoptotic process. | (30) |
### Choline Kinase Alpha (ChoKα) Inhibitors

*In vitro* and *in vivo* anticancer evaluation of the combination of ChoKα inhibitors with 5-FU in colorectal cancer cell lines and xenograft models.

- ChoKα inhibitors modulated the metabolism of 5-FU, which resulted in synergistic anticancer effects under *in vitro* and *in vivo* conditions.

### CRC

*In vitro* combinatorial treatment of 5-FU with CRC in colorectal cancer cells.

- CRC mediated apoptosis was shown in colorectal cancer cells through mitochondrial degeneration and cytochrome c release.
- In addition, CRC potentiated 5-FU-induced expression or cleavage of pro-apoptotic proteins (caspase-8, -9, -3, PARP and Bax), and down-regulated anti-apoptotic (BclxL) and proliferative (cyclin D1) proteins.

### Dichloroacetate (DCA)

*In vitro* anti-tumor evaluation with 5-FU against a series of colorectal cancer cells.

- DCA exhibited synergistic antiproliferation in combination with 5-FU against colon cancer cells.

### ATO

Phase I clinical trial of patients with metastatic, refractory colorectal cancers.

- There was significant reduction in the TS mRNA level was observed in the peripheral blood mononuclear cells of patients treated with the combination of drugs.

| Table 1.1 | Depicts a recent literature review about the drug combination studies carried out to improve the 5-FU resistance in gastrointestinal cancers including the cancers of colon and hepatic cancers (HCC) (30, 34-42). |

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(39) Choline kinase alpha (ChoKα) inhibitors modulated the metabolism of 5-FU, which resulted in synergistic anticancer effects under *in vitro* and *in vivo* conditions.

(40) CRC mediated apoptosis was shown in colorectal cancer cells through mitochondrial degeneration and cytochrome c release.

(41) Dichloroacetate (DCA) exhibited synergistic antiproliferation in combination with 5-FU against colon cancer cells.

(42) ATO Phase I clinical trial of patients with metastatic, refractory colorectal cancers.
1.2.5 Need of alternative approaches to improve the efficacy of chemotherapy in colon cancer

Despite the use of surgical resection and aggressive chemotherapy, nearly 50% of patients with colorectal carcinoma develop recurrent disease, highlighting the need for improved therapies (43). Surgery and subsequent chemotherapy can cure over 75% of colon cancer patients, but more than 30% of these patients develop new neoplastic polyps and 10% progress to frank second malignancy (44-47). The risk of second malignancy is higher for microsatellite instable tumor (45, 48). Also metastatic colorectal cancer has poor prognosis, with 5 year survival of less than 10% (43, 45). As a result of great efforts being made on improving the chemotherapeutic interventions for metastatic colon cancer, the median survival has been improved to over 20 months in this group of patients (45, 49). However, this has lead to additional toxicities, some of which are even fatal. Thus the rationale of a non-toxic agent that could improve the current chemotherapeutic regimen would therefore be highly desirable.

The unwanted toxic side effects of 5-FU due to the non selective exposure and poor bioavailability (leads to the use of over dose) could be addressed by entrapping the drug; 5-FU in a nanocarrier system. The drug entrapment in a carrier system will improve the bioavailability of 5-FU by; improved plasma drug concentration time profile, which in turn improves the efficacy of chemo treatment. The efficacy of 5-FU could also be improved by combinatorial approach. In combinatorial approach, either 5-FU in combination with other chemo drugs or 5-FU in combination with anticancer phytochemicals such as CRC could be used. The combinatorial approaches with anticancer phytochemicals are advantageous in terms of the following important points,

- Anticancer phytochemicals inhibits multiple cancer survival pathways
- Less harmful to the patient than the conventional chemo drugs

Based on this, the current thesis work has been framed to evaluate the combinatorial anticancer effects of 5-FU and curcumin(CRC) released from the
nanoformulations along with the basic pharmacokinetic studies to evaluate the bioavailability of CRC and 5-FU in comparison with the bare drugs.

1.2.6 Nanoencapsulation

Nanoparticle drug delivery systems are nanometeric carriers used to deliver drugs or biomolecules. Generally, nanometeric carriers also comprise sub-micro particles with size below 1000 nm and with various morphologies, including nanospheres, nanocapsules, nanomicelles, nanoliposomes, and nanodrugs, etc (50-52). Nanoparticle drug delivery systems have outstanding advantages (50, 51, 53-58).

The important features of nanomaterials includes

- They can pass through the smallest capillary vessels because of their ultra-tiny volume and avoid rapid clearance by phagocytes so that their duration in bloodstream is greatly prolonged (50, 59).
- They can penetrate cells and tissue gap to arrive at target organs such as liver, spleen, lung, spinal cord and lymph (50, 59).
- They could show controlled release properties due to the biodegradability, pH, ion and/or temperature sensibility of the materials (50, 59).
- They can improve the utility and reduce the toxic side effects of the drug (50, 59).
- As drug delivery systems, the nanoparticles can entrap drugs or biomolecules into their interior structures and/or absorb drugs or biomolecules onto their exterior surfaces (50, 59).
- High surface to volume ratio that is much larger than that of other particles.
- Nanoparticles are having large functionalities which can bind/conjugate/adsorb therapeutic agents such as drugs/proteins/ etc.
- Nanoparticles are characterized by their ability of enhanced permeability and retention effect (EPR) (60-65).

EPR effects helps in the accumulation of drug loaded NPs more in the tumor tissues in comparison with the normal tissues. The EPR effect is a unique phenomenon of solid tumors related to their anatomical and pathophysiological differences from normal tissues. These unique pathophysiological characteristics of
tumor vessels enable macromolecules and nanoparticles, to selectively accumulate in tumor tissues. Thus the permeation and retention of the drug loaded nanoparticles will be enhanced in the tumor tissues due to their nanosize (66-70). These features are called the EPR effect, which constitutes an important mechanism by which macromolecules and nanoparticles selectively accumulate in the tumor interstitium.

1.2.7 Nanoencapsulation of 5-FU

Recent literature described the application potential of 5-FU encapsulated nanoparticles in targeted and non-targeted anticancer applications. Additionally these works described the improved efficacy of encapsulated 5-FU unlike the bare drug under *in vitro* (71-95) and *in vivo* conditions (96-105). A few of them include; 5-FU loaded calcium carbonate mineralized nanoparticles for the intracellular breast cancer delivery (81), 5-FU-loaded poly (ε-caprolactone) nanoparticles for drug resistant colon cancers (82), 5-FU loaded magnetoliposome nanoparticles for combined antitumor therapy (83), 5-FU loaded chitin nanogels for melanoma (85), 5-FU loaded pH-responsive liposomal formulations for colon cancer (86), 5-FU loaded chitosan-coated magnetic nanoparticles for lung cancer (87), 5-FU loaded oleic acid-pluronic-coated iron oxide nanoparticles for pancreatic cancer (88), 5-FU loaded fibrinogen nanoparticles for the *in vitro* anticancer applications (89), thermo and pH responsive 5-FU encapsulated chitosan-graft-poly (N-isopropylacrylamide) nanoparticles for the *in vitro* prostate, oral and breast cancer studies (90), and 5-FU encapsulated magnetic pectin nanoparticles for the *in vitro* anticancer application towards colon, liver and pancreatic cancers (91).

A few of the targeted drug delivery applications include SM5-1 (a humanized mouse antibody has a high binding specificity for membrane proteins over expressed HCC, melanoma and breast cancer) conjugated 5-FU loaded PLGA nanoparticles for hepatocellular carcinoma (106), translocator (TSPO) protein conjugated 5-FU loaded PLGA nanoparticles for glioma (92), 5-FU encapsulated polyethylene glycol modified folate-functionalized poly (amido amine) dendrimers for oral cancer (93), 5-FU encapsulated folic acid functionalised casein-gold nanoparticles for breast cancer (94), 5-FU encapsulated folic acid-conjugated PLGA nanoparticles for colon caner
(94), and 5-FU encapsulated pectin based nanoparticles for hepatocellular carcinoma (95) applications. In conclusion the nanoencapsulation of 5-FU altered the properties of 5-FU as discussed below;

- Sustained \textit{in vitro} drug release profile
- Improved cellular internalisation
- Improved \textit{in vitro} antitumor effects
- Protects the 5-FU against clearance
- Improved the \textit{in vivo} pharmacokinetic profile
- Reduced the systemic exposure of 5-FU in the \textit{in vivo} conditions.

1.2.8 Advantages of combinatorial approach of 5-FU with CRC in colon cancer treatment

In colon cancer chemo treatment, the combinatorial approach of 5-FU with anticancer phytochemicals such as CRC can improve the efficacy of colon cancer treatment through the inhibition of multiple cancer cell survival pathways, dose reduction, and enhanced chemo sensitivity of tumor cells to many kinds of chemotherapeutic drugs including 5-FU. CRC, a principal bioactive component of \textit{Curcuma longa} (turmeric), represents one of the most investigated anticancer phytochemicals. There are 3 major curcuminoids that constitute CRC: (curcumin I, 75%), demethoxycurcumin (curcumin II, 20%), and bisdemethoxycurcumin (curcumin III, 5%, Fig. 1.2) (106-108). Last 2 decades of research on CRC has shown its potent antioxidant, anti-inflammatory, antiproliferative, antimetastatic, antiangiogenic, antidiabetic, hepatoprotective, antiatherosclerotic, antithrombotic, and antiarthritic properties in cell culture and animal studies (107, 108).
Figure 1.2. Depicts the structures of the three curcuminoids as components of CRC (107).

CRC exerts its anticancer effects towards many kinds of cancers including the cancer of colon and rectum under *in vitro* and *in vivo* conditions as depicted in Fig.1.3 (108-125). Various research works on cell culture using CRC have shown its potential apoptosis induction through the inhibition of various intracellular transcription factors and secondary messengers such as nuclear factor-kappa (NF-κB), activator protein 1 (AP-1), c-Jun, the JAK-STAT pathway, and various others (106, 108, 110, 126). CRC exhibits potent anti-inflammatory activity through the inhibition of IκB kinase required for the activation of NF-κB, an important transcriptional regulator of inflammatory pathways involved in carcinogenesis and various other pathologic conditions (106, 120, 127-130).
Figure 1.3. Depicts the anticancer effect of CRC towards various cancer cells (109, 126).

Also the results had demonstrated that the CRC treatment inhibits cyclooxygenase-2 (COX-2) expression and activity, leading to a reduction in prostaglandin synthesis and loss of cancer cell growth (131-136). Literatures were reporting the potential enhancement of anticancer efficacy of 5-FU with CRC under in vitro conditions through the inhibition of COX-2 in the mRNA and protein level. CRC being a COX-2 inhibitor, reduces the expression of COX-2 in colon cancer, which further improves the activity of 5-FU through the inhibition of thymydylate synthase (137-141).

Despite these advantages, CRC possesses poor water solubility along with limited bioavailability, which makes it a class II drug in the Biopharmaceutics Classification System (107,142-144). Furthermore its rapid intestinal and hepatic metabolism leads to the loss of 60 to 70% of an oral dose of CRC through feces (106, 145,146). Studies by Pan and colleagues showed that after intraperitoneal administration of 0.1 g/Kg CRC to mice, only about 2.25 μg/ml reached the plasma within 15 minutes which rapidly drops down to 0.35 μg/ml after 1 hour (107, 145) (Table 1.2).
CRC administration through the intravenous route results in 50% elimination through bile within 5 hours (106,150,151). In clinical studies, high doses of orally administered CRC (8-12g/daily) resulted in very low plasma CRC concentrations (<1 μg/ml), levels that were not high enough to exert any significant pharmacologic or therapeutic activity (107, 143). CRC undergoes rapid metabolism in the intestine (106, 152, 153) and liver to form various active and inactive metabolic products that are further converted into excretable glucuronide and sulfate conjugates.

These limitations lead to the limited success of CRC in various animal and clinical studies. Researchers were trying to improve the bioavailability of CRC through the use of absorption enhancers such as piperine (107, 147, 154) or by the use of novel drug delivery systems such as micelles(106, 155), liposomal vesicles(156), nanoparticles (122, 123, 157, 158), nanoemulsions (106, 159), phospholipid complexes(106, 160), and polymeric implants (107). Out of these, the technique of nanoencapsulation is more advantageous in cancer research, in terms of EPR effect, higher drug loading, improved pharmacokinetic properties and ease of functionalisation of the developed nanomedicines (60-65).

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<thead>
<tr>
<th>Animal</th>
<th>Route</th>
<th>Dose</th>
<th>Conclusions</th>
<th>References</th>
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<tbody>
<tr>
<td>Rat</td>
<td>Oral</td>
<td>1g/Kg</td>
<td>Poor absorption, 75% excreted in feces</td>
<td>(147)</td>
</tr>
<tr>
<td>Rat</td>
<td>Oral</td>
<td>2% Diet</td>
<td>12nmol/l in plasma</td>
<td>(148)</td>
</tr>
<tr>
<td>Mice</td>
<td>Intraperitoneal</td>
<td>100mg/Kg</td>
<td>2.25μg/ml in 15 minutes, Cleared within 3 hours</td>
<td>(145)</td>
</tr>
<tr>
<td>Rat</td>
<td>Intravenous</td>
<td>40mg/Kg</td>
<td>Cleared within 1 hour</td>
<td>(149)</td>
</tr>
</tbody>
</table>

Table 1.2. Depicts the preclinical studies reported with CRC (106).
1.2.9 Combinatorial approach of 5-FU with CRC

Du B and co-workers proved the synergistic anticancer effects of the concurrent use of 5-FU with CRC through the decreased expression of COX-2 protein in colon cancer; HT 29 cells (140). The potential synergistic anticancer effects of 5-FU with CRC towards gastric carcinoma cell line (AGS) was proven by Koo et al., 2004 (142). Kim and co-workers proved the dose dependent reduction in the growth of colon cancer cells after the concurrent use of 5-FU and CRC (161). Fang et al reported that the combination of CRC with 5-FU can enhance the sensitivity of antitumor agents towards cancer through the suppression of nuclear factor-kappa β (NF-kβ) (162). Srimuangwong et al., proved the synergistic anticancer effects of hexahydrocurcumin with 5-FU through the inhibition of COX-2 protein (138). Later on the same group evaluated the in vivo anticancer efficacy of hexahydrocurcumin with 5-FU on dimethylhydrazine-induced colon cancer in rats. The results proved the suppression of the growth of colorectal cancer by the use of hexahydrocurcumin combined with 5-FU without any side effects (139). Mehdi et al., 2013 also proved the effectiveness of the combinatorial treatment of CRC with conventional chemotherapeutic agents such as 5-FU towards chemo resistant colon cancer cells (40).

Recently Balasubramanian et al., in 2014 developed 5-FU and CRC encapsulated PLGA-magnetic particles conjugated with transferrin and folic acid. The cytotoxicity results proved the dose-dependent and pH-responsive antitumor effects of the developed nanoparticles towards cancer cells. Thus the research pinpoints the use of dual drug loaded system of 5-FU and CRC along with multifunctionalities for efficient cancer treatment (163). In another work, the synergistic anticancer effects of CRC with 5-FU was addressed along with nanoencapsulation to improve the poor bioavailability issues of CRC and 5-FU. The developed CRC encapsulated solid lipid nanoparticles and 5-FU encapsulated layered double hydroxides produced synergistic anticancer effects in hepatocarcinoma cells (SMMC-7721) (164).
1.2.10 Nanoencapsulation of CRC

The advent of nanotechnology has been exploited for the development of various nanoparticulate drug delivery systems that can enable formulation and delivery of CRC (165-184). These nanoformulations were developed in different carriers. A few examples include poly (2-hydroxyethyl methacrylate) [PHEMA] nanoparticles (165), composite nanosystem of N-isopropylacrylamide (NIPAAm), vinylpyrrolidone (VP), and acrylic acid (AA) (166), poloxamer 188 coated poly (butyl) cyanoacrylate (PBCA) nanoparticles(167,168), PNIPAAm and PLGA nanoparticles (169, 170), lipid-nanospheres (171), PLGA nanoparticles (172), and pluronic F127 stabilised PLGA (173) etc. These delivery systems have gained immense popularity in the last decade due to their;

- Potential to improve the therapeutic index of the encapsulated drugs either by;
  - By protecting them from enzymatic degradation (185).
  - By altering their pharmacokinetics (186).
  - By blunting their toxicity (187).
  - By providing controlled release over extended periods of time (188).

The pharmacokinetics, biodistribution and therapeutic efficacy of different CRC nanoformulations have been investigated in many studies in order to get insight into the potential value of these systems for the treatment of different diseases including cancer. A few reports include a CRC nano-emulsion of PEG 600 and Cremophor EL with a 40 fold increase in the maximum concentration (Cmax) and a 10 fold increase in area under the curve (AUC) in mice(189), CRC encapsulated solid lipid nanoparticles with 1.25 fold increased bioavailability in rats (intravenous administration) (190), CRC encapsulated PLGA and PLGA-polyethylene glycol (PEG) (PLGA-PEG) blend nanoparticles with a 15.6 and 55.4 fold oral bioavailability(191), PEG-5000 modified CRC encapsulated PLGA nanoparticles with improved bioavailability and half-life(123), CRC encapsulated lauroyl sulphated chitosan with a 11.5-fold increased oral availability (192), PLGA nanoformulation with a 22-fold higher oral bioavailability (121), CRC loaded solid lipid nanoparticles (C-SLNs) with improved oral bioavailability in mice model (193),
CRC loaded PLGA nanospheres with a 9 fold increase in the oral bioavailability (194), CRC encapsulated polyvinyl pyrrolidone (PVP)-gold nanoparticles with improved bioavailability (195). CRC encapsulated glycerol monooleate and Pluronic F127 nanoparticles with a 1000 fold higher peak concentration(intravenous injection) in mice (196), CRC loaded PLGA-PEG-PLGA micelles with substantial improved AUC, mean residence time, clearance half-life and distribution half-life of CRC (intravenous injection) in mice (197,198), and CRC nanosuspension of TPGS (D-tocopheryl polyethylene glycol succinate) with a 4 and 11 fold increased AUC and mean residence compared to free CRC (199) after intravenous administration. Also CRC encapsulated system of NIPAAm, vinylpyrrolidone (VP), and acrylic acid (AA) was proven to have a greater Cmax, and AUC after the left jugular vein administration in male Sprague Dawley rats (200). The pharmacokinetics and tissue distribution studies of CRC loaded-solid lipid nanoparticles (CRC-SLNs) in mice model revealed the improved plasma concentrations of CRC in comparison with the free CRC (201). From the studies summarized above, it is evident that nanoformulations can improve the half-life and AUC of CRC. Their small size and slow release profiles contribute to the prolongation of the elimination time (202-209).

Many recent in vivo studies have demonstrated the improved antitumor effects of the CRC encapsulated nanoparticles unlike bare CRC. A few examples include the CRC encapsulated methoxy poly (ethylene glycol)-polycaprolactone (mPEG-PCL) polymeric micelles for subcutaneous LL/2 pulmonary carcinoma (210), CRC encapsulated dendrimeric glycol ester in mice model (211), CRC-loaded lipid nanocapsules in glioma (184), CRC loaded g-cyclodextrin liposomal nanoparticles for osteosarcoma (212), and CRC loaded PLGA nanoparticles for hepatocellular carcinoma (213) etc.

1.2.11 Chitosan based polymeric materials as drug delivery system

Chitosan based polymeric materials are characterized by their high number of reactive groups, a wide range of molecular weight (Mw), and varying chemical composition, which contribute to their diversity in structure and property. These materials are characterized by their;
Ease of chemical/biochemical modification resulting from its various derivable groups resulting in the formation of polysaccharide derivatives (50, 214)

- High stability (50, 214)
- Safety and non-toxicity (50, 214)
- Hydrophilicity (50, 214)
- Biodegradable nature (50, 214)

All these qualities provide chitosan based materials as promising future biomaterials. Recently a lot of studies have been conducted on chitosan and their derivatives for their potential application as drug delivery systems; especially as nanoparticle drug delivery systems (50, 214-217). Chitosan is a nontoxic and biocompatible polysaccharide derived from the shells of crustaceans, insects and lobsters (218-222). A lot of studies have described the application potential of chitosan based systems as promising delivery systems for different therapeutic agents. The safety of chitosan has been demonstrated in both animals and humans (218, 223-228).

Chitosan obtained from chitin and both chitin and chitosan are linear polysaccharides, comprised of two monomeric units namely N-acetyl-2-amino-2-deoxy-D-glucose (N-acetylated groups) and 2-amino-2-deoxy-D-glucose residues (N-deacetylated groups, amino groups). Chitin samples contain low amount of 2-amino-2-deoxy-D-glucose and hence it is less soluble in acidic solvents, whereas chitosan samples contain lesser number of N-acetyl-2-amino-2-deoxy-D-glucose and hence it is soluble in acidic solvents (218, 221, 222, 229-231). The structure of chitin and chitosan is depicted in the following Fig.1.4.
Figure 1.4. Depicts the chemical structure of chitin and chitosan.

In addition to protecting the loaded therapeutics against acidic denaturation and enzymatic degradation, chitosan based materials exhibit mucoadhesive feature, capable of prolonging their residence time in the small intestine (218, 227, 228). It can also mediate the opening of tight junctions (TJs) between epithelial cells reversibly, thus facilitating the paracellular transport of hydrophilic macromolecules (218, 232-234). Because of these important features of chitosan, many studies have focused on the usage of chitosan based nanoparticles in oral drug delivery, where the researchers have addressed the problem of oral bioavailability of macromolecular agents (218, 235-238). In brief the important features of chitosan and its derivatives based materials to be used in drug delivery or biomedical applications can be summarized as follows (218, 239-249).

- Biodegradability
- Biocompatibility
- Non-toxicity
- Ease of functionalisation; e.g.: chemical modification
- Mucoadhesion (Important in oral drug delivery)
- The degradation products are non-toxic
Chapter 1

- Readily available
- Low cost

1.2.11.1 Need for the chemical modification of chitosan

The important structural features of chitin/chitosan such as degree of acetylation (DA), degree of substitution (DS) and molecular weight (Mw) greatly influence its properties such as solubility, physiological activities (250-256), chemical reactivity and biodegradability (250, 257). Chitosan can be dissolved in aqueous solutions with a pH < 6.5; the required neutralization prior to biological applications may result in the change in the shape and size of the material. Therefore, chemical modification of both chitin and chitosan to increase their solubility in common solvents is obligatory to maximize their utility. The aqueous solubility of chitosan can be improved by chemical modification methods (218, 228, 250-253). The important chemical modification methods include quaternization, thiolation, carboxylation, alkylation, acylation, PEGylation and graft copolymerization (209, 220, 221, 230, 232, 240, 241, 250, 258-261). The nontoxicity, biocompatibility and biodegradability of the chemically modified chitosan conjugates were comparable with the parent compound; chitosan. Thus chemically modified chitosan also gained importance as novel excipient in developing controlled, mucoadhesive delivery systems of various dosage forms (262-264).

Current thesis work explores the application potential of two chemically modified chitosan derivatives. These include carboxymethylfunctionalised (N, O-carboxymethyl chitosan; N, O-CMC; Fig 1.5 A) and thioglycolic acid functionalised chitosan (thiolated chitosan; TCS Fig 1.5 B). TCS as well as the N, O-CMC nanoparticles were evaluated as the carrier systems for both 5-FU and CRC. Since both the TCS and N, O-CMC possess different chemical functionalities, the final drug loaded formulations will be different in terms of drug entrapment and subsequently the rest of the physicochemical and biological properties.
1.2.11.2 \textit{N, O-CMC nanoparticles (N, O-CMC NPs)}

Carboxymethyl chitosan nanoparticles can act as efficient drug carrier systems. Recent literatures were suggesting the potential of carboxymethyl chitosan based nanoparticles systems in targeted as well as non targeted drug delivery applications. The important features of carboxymethyl derivatives of chitosan (CMC) include:

- Improved functionality
- Biodegradability
- Biocompatibility
- Nontoxicity
- Anionic in nature
- Water soluble

Because of these excellent properties, CMC found applications in pharmaceutical, veterinary medicine, biomedical and environmental fields (218, 250, 265-280). The synthesis involves the carboxymethylation of chitosan using monochloroacetic acid in alkaline medium. \textit{N, O-CMC} is hydrophilic and a typical kind of amphoteric polyelectrolyte (218, 281, 282).

1.2.11.3 \textit{N, O-CMC NPs as drug delivery systems}

Carboxymethyl chitosan nanoparticles can act as efficient drug carrier systems. Recent literatures were suggesting the potential of carboxymethyl chitosan based nanoparticles systems in targeted as well as non targeted drug delivery
applications (283-308). Their unique properties include their ability to modify the properties of the bare drug. The important modified features are depicted below,

- Protection of the drug and reduces its degradation
- Improved redispersibility of hydrophobic drugs in physiological solutions
- Improved cellular uptake
- Sustained and controlled drug release profile
- Ease of functionalisation with multiple agents for targeting and imaging applications.
- Modified pharmacokinetic and biodistribution profile

1.2.11.4 Thiol functionalized chitosan nanoparticles (TCS NPs)

Thiol modified chitosans were obtained by the covalent coupling of the sulphhydryl bearing agents such as thioglycolic acid (218,309), and glutathione (218, 311-314), on to the back bone of chitosan. Thiolation can additionally be carried out by the ring opening of 2-iminothiolane or a direct imidooester reaction of isopropyl-S-acetyltioacetimidate (218, 249, 315). In comparison with the unmodified chitosan, thiolated chitosan derivatives were characterized by their improved functionalities and mucoadhesive strength, the latter increases the strength of mucoadhesion with the intestinal mucosa. This property of thiolated chitosan derivatives makes it usable in oral drug delivery applications (218, 316-331).

The covalent modification of chitosan results in the formation of chitosan-thioglycolic acid (CS-TGA) (218, 309), chitosan-cysteine (CS-Cys) (218, 325), chitosan-glutathione (218, 311), chitosan-4-thio butyl-amidine (CS-TBA) (218, 312, 313) and chitosan-thioethylamide (CS-TEA) (218, 311). The following Fig.1.6 represents the reaction scheme for the thiol functionalisation of chitosan through covalent modification.
Figure 1.6. Represents the reaction scheme for the synthesis of thiolated chitosan derivatives through covalent coupling methods: chitosan-thioglycolic acid, chitosan -cysteine, chitosan -4-thiobutyl-amidine, and chitosan-thioethylamidine (218).

The thiol functionalized chitosans have so many favorable properties when compared to the parent molecule chitosan such as permeation enhancement, amended mucoadhesion property. As the mucoadhesiveness of the polymer is significantly increased and leads to the formation of matrix, drug release from the formulation will be rate controlled. The therapeutic agent will be slowly released into the dissolution medium or in to the systemic circulation and their by allow to achieve the steady state concentration of the drug for longer duration of time which is very much essential in case of poorly soluble drugs or drugs with less plasma half life (Ex. Cefuroxime axetil, ofloxacin, ciprofloxacin, methotrexate etc) (329-332).

1.2.11.5 TCS nanoparticles as drug delivery systems

The important features which make the thiolated chitosan based nanoparticles to be used in drug delivery applications include its non-toxicity, biodegradability, increased functionalities which helps in drug encapsulation as well as ease of functionalisation with other targeting moieties, mucoadhesive properties, sustained drug release profile etc. Most of the studies have focused on the oral administration
applications to improve the bioavailability of poorly bioavailable drugs. A number of reports were proving the application potential of thiol functionalized chitosan based nanoparticles systems in the field of drug delivery (333-353). A few of them are depicted below.

Talaei et al., 2011 evaluated the potential of NAC-C (N-acetyl cysteine-chitosan) and NAP-C (N-acetyl penicillamine-chitosan) nanoparticles as a targeted drug delivery system for breast cancer using antisense oligonucleotide (ASOND) as the targeting ligand and doxorubicin (DOX) as the chemotherapeutic agent. The ASOND-loaded thiolated particles significantly suppressed EGFR gene expression in breast cancer (T47D) cells compared with ASOND-loaded chitosan particles and downregulated EGFR protein expression in cells (333). Saboktakin et al., in 2010 evaluated the ability of docetaxel (DTX) loaded thiolated chitosan nanoparticles for enhancing its oral bioavailability. The pharmacokinetic analysis results in Wistar rats proved the 9 fold increase in the half life of DTX released from the DTX-loaded NPs unlike the DTX control. Also the oral bioavailability of DTX was increased to 68.9% for DTX-loaded nanoparticles compared to 6.5% for positive control (335). In another study Wang et al. showed that insulin-loaded thiolated chitosan nanoparticles substantially improved the absorption of insulin across nasal mucosa as compared to non-thiolated chitosan nanoparticles as well as soluble chitosan (336). Saremi et al., 2013 showed the improved plasma half-life of DTX loaded thiolated chitosan nanoparticles in Wistar rats in comparison to that of control DTX after IV injection (337).

1.3 Thesis Scope

The effectiveness of 5-FU as a chemo drug for colon cancer can be improved by two approaches; nanoencapsulation and combinatorial treatment. The former involves the entrapment of 5-FU in a non-toxic nanobased carrier system, which reduces the non-selective exposure of 5-FU and improves its plasma half life, the latter involves the use of non-toxic COX-2 inhibitors such as CRC in combination with 5-FU.
Our research strategy is to explore the combinatorial anticancer effects of 5-FU with CRC for improving the chemotherapeutic efficacy of 5-FU in colon cancer where in the technique of nanoencapsulation has been employed to improve the bioavailability of both drugs and to reduce the non-selective exposure of 5-FU (Fig 1.7). Through the study, we are trying to improve the anticancer efficacy of 5-FU via combinatorial approach with CRC, along with the drawbacks of the drugs; 5-FU and CRC were addressed by nanoencapsulation technique. The nanoencapsulation has been achieved by two chemically modified chitosan derivatives having different functionality; carboxymethyl functionalised chitosans (\(N, O\)-CMC) and thiolfunctionalised chitosans (TCS). Both the carrier systems (\(N, O\)-CMC and TCS) were evaluated individually for the drug encapsulation, \emph{in vitro} drug release profile, hemocompatibility, \emph{in vitro} combinatorial anticancer effects and \emph{in vivo} pharmacokinetics studies. The rationale for taking these two carrier systems is to analyze the drug encapsulation potential of the nanoparticles of chemically modified systems of chitosan. The solubility issues of chitosan in water could be addressed by the use of \(N, O\)-CMC and TCS, since these chemically modified derivatives are soluble in water. Thus the systems; 5-FU loaded \(N, O\)-CMC nanoparticles; 5-FU-\(N, O\)-CMC NPs / CRC-loaded \(N, O\)-CMC nanoparticles; CRC-\(N, O\)-CMC NPs (system 1) and 5-FU loaded thiolated chitosan nanoparticles NPs; 5-FU-TCS NPs/CRC loaded thiolated chitosan nanoparticles; CRC-TCS NPs (system 2) were made separately and characterized. The combinatorial anticancer effects of the nanoformulations were proven in colon cancer cells (HT 29) by MTT, live dead, mitochondrial membrane potential measurements and cell cycle analysis(through propidium iodide; PI staining) assays. The animal experiments were performed to analyze the pharmacokinetic profile of system 1 and 2 in Swiss Albino mouse model.
Figure 1.7. Depicts the research strategy for the present thesis work.

Based on all these background, the major research questions, hypothesis and objectives for the research study are formulated.

1.4 Research questions

1. How efficiently $N,O$-CMC nanoparticles and TCS NPs can act as efficient carrier system for hydrophobic phytochemical ‘CRC’ and hydrophilic chemodrug ‘5-FU’

2. What would be the difference in anticancer effects of CRC-$N,O$-CMC-NPs and 5-FU-$N,O$-CMC-NPs (system 1) and CRC-TCS-NPs and 5-FU-TCS NPs(system 2) and in combination compared to the individual formulations in colon cancer (HT 29) cells

3. What would be the plasma concentration Vs time profile of 5-FU and CRC following a simultaneous intravenous administration of the individual nanoformulations (5-FU-$N$, $O$-CMC NPs & CRC-$N$, $O$-CMC NPs and 5-FU-TCS NPs & CRC-TCS NPs) over the bare drugs in Swiss Albino mice.

1.5 Hypothesis

1. $N$, $O$-CMC NPs/ TCS NPs interact with CRC and 5-FU through electrostatic interaction/hydrogen bonding will result in efficient drug loading.
2. Enhanced cell death will be observed when the nanoformulations are administered in combination compared to the independent nanoformulations (system 1 and 2).

3. Based on the *in vitro* drug release profile, we are hypothesizing that the nanoformulations will release 5-FU and CRC in a sustained manner for an extended time period in comparison with the bare 5-FU and CRC in blood.

### 1.6 Objective of this research work

The major objectives of the study include the extensive *in vitro* combinatorial anticancer effects of CRC and 5-FU released from the nanoformulations(system 1 and 2), followed by the *in vivo* pharmacokinetic profile of the individual as well as the combinatorial nanoformulations in Swiss Albino mouse model. The nanoformulations of CRC and 5-FU were made independently with chemically modified chitosan derivatives as the carriers. The chemically modified chitosan derivatives include N, O-CMC and TCS. Thus the major objectives of the present thesis can be explained as follows,

- To evaluate the *in vitro* combinatorial anticancer effects of 5-FU and CRC from its independent nanoformulations
  - 5-FU- N, O-CMC NPs and CRC-N, O-CMC NPs (system 1)
  - 5-FU-TCS NPs and CRC-TCS NPs (system 2)

- To evaluate the *in vivo* pharmacokinetics of the nanoformulations of 5-FU and CRC independently and in combination.
  - 5-FU-N, O-CMC NPs and CRC-N, O-CMC NPs (system 1)
  - 5-FU-TCS NPs and CRC-TCS NPs (system 2)

### 1.6.1 The specific objectives of the study

1. Understanding the feasibility of chemically modifying chitosan in to N, O-CMC and TCS and its nanoparticles
   - To develop and characterize N, O-CMC and TCS from chitosan.
   - To develop and characterize N, O-CMC and TCS nanoparticles.
Chapter 1

• To evaluate the *in vitro* cytocompatibility of *N, O*-CMC and TCS nanoparticles using MTT assay

• To evaluate the *in vitro* hemocompatibility of *N, O*-CMC and TCS nanoparticles using hemolysis and coagulation assays

2. To evaluate the possibility of encapsulating 5-FU and CRC individually in both the nanocarrier systems (*N, O*-CMC and TCS)
   - To develop and characterize 5-FU loaded *N, O*-CMC nanoparticles (*5-FU-N, O*-CMC NPs) and CRC loaded *N, O*-CMC nanoparticles (*CRC- N, O*-CMC NPs).
   - To develop and characterize 5-FU loaded thiolated chitosan nanoparticles (*5-FU-TCS NPs*) and CRC loaded thiolated chitosan nanoparticles (*CRC-TCS NPs*).

3. To evaluate the hemocompatibility of the developed nanoformulations
   - Hemolysis and coagulation assays of *5-FU-N, O*-CMC NPs, *CRC- N, O*-CMC NPs, *5-FU-TCS NPs* and CRC-TCS NPs.

4. To evaluate the *in vitro* drug release profile of the drug loaded nanoformulations

5. To measure and quantify the *in vitro* combinatorial anticancer effects of both systems (system 1 and 2) against colon cancer cells
   - The combinatorial anticancer effects were quantified and confirmed using MTT, live dead, mitochondrial membrane potential and cell cycle analysis measurements

6. To evaluate the *in vivo* pharmacokinetics of the drug loaded nanoparticles in Swiss Albino mouse model up to 72 hours.

1.7 Thesis outline

In Chapter 1 the first part is the introduction of the thesis and the second part is the comprehensive review of relevant literature for this thesis. Also the research questions and hypothesis were formulated based on the literature review.

In Chapter 2 the materials and experimental methods used in the research study of this thesis is described.
In Chapter 3 the results and discussion of the research studies conducted are described. Finally in Chapter 4, the work as described in this thesis is summarized and the future perspectives are discussed.