DISCUSSION

The incidence and mortality rates due to colorectal cancers have been increasing in a steady manner in recent times (2). Globally CRC is the 4th most common cause of deaths from cancer and is accounting for the mortality of ~ 608,000 (8% of all cancer deaths) individuals every year (2). Although the age adjusted incidence rates of CRC in India are very close to the lowest rates in the world, recent population based trend studies are demonstrating a rising trend of CRC in India (3). In addition according to GLOBOCAN 2008 (an international agency for research on cancer) cancer data base, about 25,000 individuals succum to CRC every year in India (2).

Colorectal cancers develop slowly over a period of 10 to 15 years (92). The tumor typically begins as a noncancerous polyp. Certain kinds of polyps, called adenomatous polyps or adenomas, are the most likely to become cancers, though fewer than 10% of adenomas progress to cancer (93). About 96% of colorectal cancers are adenocarcinomas, which evolve from glandular tissue (94). Major risk factors that have proven to induce CRC include (a) personal or family history of colorectal cancer; (b) chronic inflammatory bowel diseases; (c) obesity; (d) consumption of processed meat / red meat; and (e) smoking, and alcohol drinking (3). In addition physical inactivity is also found to induce CRC (4). Surveillance, Epidemiology, and End Results (SEER) system staged CRC into in situ, local, regional and distant forms (http://www.cancer.org/acs/groups/content/@epidemiologysurveillance/documents/document/acspc-028323.pdf) (95). Whereas in situ forms are preinvasive lesions not invading the wall of the colon or rectum, the local forms are grown into the wall of the colon and rectum, but not extended through the wall to invade the nearby tissues (95). The regional and distant forms are invasive CRCs with the ability to invade nearby tissues (including nearby lymph nodes) as well as other parts of the body (primarily lung and liver) respectively (95).

Currently, only 3 targeted monoclonal antibody therapies have been approved by the United States Food and Drug Administration (USFDA) to treat distant forms of CRC (96), (97). Whereas bevacizumab blocks the angiogenesis by inhibiting VEGF-A, cetuximab and panitumumab block the effects of EGFR (8). However, recent studies showed that tumors with certain genetic mutations (like KRAS gene, whose product is a component of the EGFR signaling pathway) do not benefit from these treatments, especially with cetuximab or panitumumab (98). In addition, these treatments found effective only in patients harboring activated EGFR (in case of cetuximab or panitumumab) or in tumors where VEGF-A expression is critical for the
tumor growth and survival (99). Furthermore, recently completed clinical trials evaluating the efficacy of cetuximab alone or in combination with chemotherapeutics oxaliplatin plus fluoropyrimidine like capecitabine or 5FU plus leucovorin (reduced folic acid) found no clinical benefit from the treatment (http://www.cancer.gov/clinicaltrials/results/summary/2011/COIN0811). Adding to these drug failures, knowledge about the markers representing the drug resistance is also meager. These lacunae along with the poor prognosis, quiet often associated with metastatic CRC, underlines the importance of developing efficacious strategies for early interventions. Therefore, there is a urgent need for the identification of (a) key proteins regulating the development of drug resistance in colorectal cancers using clinical specimens; and develop (b) potent drugs for effectively killing the CRC tumors.

Hence, in this study we have identified Nrf2 as a key therapeutic target by measuring its expression in cell line representing colorectal carcinoma ie., HCT-116; and developed novel small molecule inhibitor for retarding the Nrf2 expressing CRC cells. As a proof of principle, effect of targeting Nrf2 using small interfering RNAs (siRNAs) on HCT-116 cell growth was measured and demonstrated inhibition of cell proliferation upon decreasing Nrf2 levels. Recent studies have identified nuclear factor (erythroid-derived 2)-like 2, also known as NFE2L2 or Nrf2 as a key therapeutic target for inhibiting different cancers (17). The Nuclear factor erythroid 2-related factor 2 (Nrf2)/ Kelch-like ECH-associated protein 1 (Keap1) system represents an important mechanism by which mammalian cells can sense and adapt to chemical and oxidative stresses (15). Normally, Keap1 targets Nrf2 for ubiquitylation, leading to its proteasomal degradation (25). However, in response to chemical or oxidative stress, the interaction between Nrf2 and Keap1 is perturbed, resulting in the stabilization and nuclear accumulation of Nrf2 (28). Nrf2 localised in the nucleus interacts with antioxidant response elements in the promoter regions of a plethora of genes coding for phase 2 detoxifying enzymes (e.g. glutathione-S-transferases (GST) and NAD(P)H quinone oxidoreductase (NQO1), antioxidant proteins (e.g. glutathione synthetic enzymes) and transporters (e.g. ABCC2, ABCC3, ABCG2 and νc - subunit) (16). Elevated Nrf2 levels that have been observed in head and neck (100), larynx (101) and lung cancer (102) found to induce cancer cell proliferation and resistance to oxidative stress and chemotherapy. A very recent study measuring the expression of Nrf2 in CRC tumors of 149 cases has reported elevated expression (using IHC) in 99 cases (103). In addition, authors of this study
has correlated and demonstrated an inverse relation between Nrf2 expression and patient survival time (103).

Supporting this observation, results of preclinical studies showed suppression of NRF2 expression reduced tumor cell proliferation and sensitized cells to chemotherapeutic agents treatment (34). Therefore, targeted inhibition of Nrf2 in cell lines already rich in Nrf2 is a viable strategy to mitigate tumor growth. Very few recent reports also showed that genetic ablation of Nrf2 using siRNA retards colorectal carcinomas in vitro and in vivo (33). However, to date no potent anti-Nrf2 agent with clinical viability has been identified. Hence, in this study an attempt was made to synthesize derivatives of tetrahydrocarbazoles (THCs) and Oxazines. The prepared derivatives were tested for inhibiting the growth of Nrf2 expressing cell lines HCT-116 and A549 (Figures 15-21). The potent derivative THC-5b was tested in mouse models for inhibiting the growth of Nrf2 expressing EAC cells in the intraperitoneal cavity (104). Administration of THC-5b retarded tumor growth compared to vehicle treated cells (Figure 36). Interestingly, analysis of the THC-5b treated EAC cells expressed high NQO1 compared to control animals (Figure 38).

While down-modulating Nrf2 is required in cancers such as CRC, upregulating Nrf2 is required to prevent the transformation of normal cells in to cancer cells (29). Highlighting this dual role, a recent systematic review by Gonzalez-Donquiles, C., et al., 2017 demonstrated that Nrf2 has a complex role in CRCs and disruption (upregulation or downregulation) of its natural level of expression promotes the genesis and progression of colorectal carcinomas (29). Therefore, pharmacological agents modulating Nrf2 levels are attractive anti-cancer agents (29). A recent study by Havermann, S., et al., 2016 showed activation of Nrf2 in HCT-116 cell line by a natural product baicalein (105). Baicalein inhibited the kinase that phosphorylates Nrf2 at Ser40 thereby prevent its degradation (105). Another activator of Nrf2 and well-explored natural product sulforaphane also inhibit HCT-116 cells growth through the inhibition of target genes HIF1α and VEGF(106). Likewise, phenolic compounds isolated from natural sources also activate Nrf2 thereby act as efficient chemopreventive agents (107). In our study the THC-5b activated Nrf2-target gene NQO1 in animals. Elevated NQO1 resulted in the inhibition of tumor growth (Figure 38). However, the mechanism of NQO1 activation and how the activated NQO1 causing tumor growth inhibition needs to be determined. A possible mechanism could be that increase in the stabilization of tumor suppressor protein p53 through its physical interaction (108).
Although chemoprevention using Nrf2 activators such as the one reported in this study, is a preferred approach, in majority of the cases it is the therapeutic strategy required to protect patients (32), as diagnosis of cancers occur in late stages in reality (109). Therefore, inhibition of elevated Nrf2 is required for cancer treatment (110). Several recent studies have demonstrated that targeted reduction of Nrf2 using pharmacological agents or genetic manipulation methods sensitized cancer cells to chemotherapeutic agents, radiation and anti-body based therapeutics (21). In this study oxazines API and COM found to inhibit the Nrf2-target gene NQO1 in a dose dependent manner thereby retarded EAC cell growth in animals (Figure 37). A recent review by Zhu, J et al., 2018 explained how chemical inhibitors of Nrf2 promote cancer cell killing (111). Whereas compounds luteolin, wogonin, mycotocin ochratoxin-A promote the degradation of Nrf2 mRNA, pharmacological agents apigenin, metformin, chrysins, 3′,4′,5′,5,7-pentamethoxyflavone and 4-methoxychalcone enhances the degradation of Nrf2 protein by promoting phosphorylation of related signaling proteins (111). Brusatol, a well known Nrf2 inhibitor, inhibits the translation of Nrf2 (41). Compounds ascorbic acid, retinoic acid, trigonelline, isoniazid prevents the nuclear import of Nrf2, which locks the Nrf2 in cytosol (111). In addition, retinoic acid and mycotocin ochratoxin-A can also prevent the binding of Nrf2 to target DNA (111). In summary, inhibition of Nrf2 signaling can be achieved at different levels using pharmacological agents. However, it is currently not known how 1,2-oxazines regulate Nrf2 activity. Results of in silico study using oxazines showed their ability to bind to Keap1 binding site of Nrf2, which might be inducing the degradation of Nrf2 (Figures 31 & 32). A study by Ansari, N., et al., 2011 studied the effect of 2-ethoxy-4,5-diphenyl-1,3-oxazine-6-one (EDPOO) against H2O2-induced cell death in rat pheochromocytoma (PC12) cells and demonstrated that EDPOO protect cells from hydrogen peroxide induced damage (49). Mechanistically, EDPOO induced the expression of Nrf2, HO1, Hsp-70 and γGCS thereby reduced the toxicity caused by hydrogen peroxide treatment (49).

In summary, results of this doctoral thesis identified a key role for Nrf2 in colorectal carcinomas, and synthesized a series of chemical compounds to modulate Nrf2 expression. Whereas THC-5b is a potent enhancer of Nrf2 target protein NQO1, the oxazines API and COM effectively reduced its activity in EAC cells. Further studies are warranted to determine the mechanisms of action of these compounds. In addition, evaluation of these compounds to inhibit the xenografts of Nrf2 expressing cell lines is warranted. Additional studies are also required to assess the selectivity of these compounds for cell lines expressing or not expressing Nrf2.