Summary and Conclusion

Inflammation is a critical component of tumor progression. Several cancers occur from the sites of infection, chronic irritation and inflammation. Also, the tumor microenvironment is largely orchestrated by inflammatory cells leading to the cell survival, proliferation and migration. Chronic inflammation and cancer share an undeniable association. Intestinal carcinogenesis is decreased by anti-inflammatory drugs while the occurrence of ulcerative colitis increases the incidence of colorectal cancer which leads to the common believe that the neoplastic transformation of the intestinal epithelium is caused by the process of chronic inflammation. There are other connections which associate ulcerative colitis and colon cancer such as the epidemiological studies which show lowered mortality from sporadic colorectal cancer and regression of adenomas in patients with Familial Adenomatous Polyposis (FAP) by the regular administration of the anti-inflammatory drugs, NSAIDs. Though cyclooxygenases are the key to both the diseases, yet the molecular basis of the association is not completely understood. The present study therefore, focussed on this relationship between ulcerative colitis and colon cancer. This was achieved by the administration of DSS to induce ulcerative colitis, and DMH to induce colon cancer and a combination of both to study the inflammation mediated colon cancer in Balb/c mice.

For the dose standardization of DMH, the animals were divided into six groups: control, 1mg/kg DMH, 10 mg/kg DMH, 20 mg/kg DMH, 30mg/kg DMH and 50mg/kg DMH. The animals showed visible lesions in the colon called the multiple plaque lesions (MPL), which are the raised or non-raised stretch of tissue visible with a sign of neoangiogenesis as compared to the adjacent non affected tissues. Our results demonstrated a positive correlation between the yield of colonic MPL and the increase in the dose of DMH in Balb/c mice in a time period of six weeks. No lesions were evident in the control, 1mg/kg and 10 mg/kg DMH groups, while 66.7% lesion incidence was seen at 20mg/kg DMH dose which increased to 100% at 30 mg/kg as well as at 50mg/kg of DMH. Also, ACF, a pre cancerous change, is visible prominently, which is now widely used as a biomarker for colon carcinogenesis studies. The present study showed a linear progression between the number of ACF and the increase in DMH dose. Furthermore, the mucosal dysplasia was 100% with the 20, 30 and 50 mg/kg DMH dose. Keeping in view, the MPL and ACF incidence, morphology, histology
and the extent of mortality, we chose the dose of 30 mg/kg of DMH for subsequent studies. Also, Celecoxib, a Cox-2 selective NSAID at a dose of 6mg/kg was found sufficient to restrain the inflammatory response as examined by the carragenan induced paw edema test. Thus, Celecoxib was used as a chemopreventive agent against the DSS induced colitis and DMH induced colon carcinoma.

For the study of ulcerative colitis associated colon carcinogenesis, the animals were divided into eight groups comprising of control, DSS, DMH, Celecoxib, DSS + DMH, DSS + Celecoxib, DMH + Celecoxib and DSS + DMH + Celecoxib. These groups were sacrificed after 6 weeks (early stage) and 18 weeks (advanced stage) of the treatment period.

In the initial stage of carcinogenesis (6 weeks), no MPL were observed in the control, DSS, Celecoxib and DSS + Celecoxib groups but 100% MPL incidence was seen in the DMH+DSS and DMH groups. MPL burden and multiplicity was also quite high in these groups. These were corrected to a significant extent with the co-administration of celecoxib in these groups which led to a sharp decrease in the MPL incidence, burden and multiplicity. Also, ACF was studied for the 6 week treatment period. It was found that maximum number of ACF existed in the DMH + DSS group followed by DMH and DSS alone group. Their number was reduced significantly when celecoxib was co-administered with DSS, DMH and DSS + DMH. Histopathologically, normal histology was observed in the control and celecoxib groups where the layer of colonic mucosa lay above submucosa and muscularis mucosa. Also, there were a large number of healthy crypts which were filled with mucus containing goblet cells. With the treatment of DSS, there was a sharp decrease in the number and size of crypts and the number of goblet cells in them along with the neutrophil infiltration. The DMH treatment led to the occurrence of dysplastic crypts with pycnotic nuclei and also hyperplasia was evident. These features were seen in the combination group of DSS + DMH with neutrophil infiltration, decreased crypt number as well as the number of hyperplastic and dysplastic crypts. The treatment with Celecoxib in these groups lowered these features and brought these towards normal cellular architechture.

In the advanced stage of carcinogenesis (18 weeks), we observed 100% tumor incidence in the DMH + DSS and DMH alone groups while no tumors/lesions were observed in the control, DSS, Celecoxib and DSS + Celecoxib groups. The co-treatment of Celecoxib with DMH and DMH + DSS led to a drastic decrease in the tumor incidence, burden and multiplicity. The study of ACF also showed their increased number with the treatment with
DSS and DMH, alone as well as in combination. The co-administration of Celecoxib lowered the number of ACF in these groups. Normal histopathology was observed in the control and celecoxib groups with normal crypts filled with goblet cells, embedded in the stroma in the mucosal layer. The administration of DSS led to the drastic reduction of the number of goblet cells in the crypts as well as the crypt size and numbers. The signs of inflammation were evident from the lymphoid aggregation of the neutrophils, high myeloperoxidase activity and the thickening of submucosa. The treatment with DMH led to the disruption of epithelial layer. Darkly stained pycnotic nuclei were abundant in the crypts with a visible loss in the mucus secreting goblet cells. Group 5 (DMH + DSS) revealed the neutrophil infiltration along with hyperplastic crypts. These effects of DSS and DMH were reverted significantly by the co-administration of Celecoxib in DSS, DMH and DSS + DMH where the number of goblet cells has been found to be increased in the crypts along with the regenerating epithelium and reduced number of pycnotic nuclei. These tissues also showed very high histochemical reactivity of mucin in the goblet cells.

The role of inflammation in carcinogenesis has long been appreciated and we presently analyzed its role by examining the expression of COX-2 enzyme. Results showed a strikingly raised expression of COX-2 in the DSS, DMH and DSS + DMH groups, which clearly indicated the role and the inducible nature of this enzyme in cancer progression. However, the treatment with celecoxib showed a decrease in the expression of COX-2 levels. We also explored the role of the house keeping COX-1 enzyme and found it to be present ubiquitously in all the groups, as expected.

Further, we explored the role of NF-κB in inflammation augmented tumorigenesis and found it to be elevated in DSS, DMH and DSS + DMH group as compared to the control, as seen by western blots and immunofluorescent analysis. The increased expression was however, downregulated with the administration of Celecoxib in these groups. Inhibitory κB (IκB) expression was lowered in these groups and was brought towards normal with the co-treatment with Celecoxib. The expression of IκB kinase (IKK) was upregulated with the treatment with DSS and DMH, although the co-administration of Celecoxib led to the downregulation of these levels towards normal. Therefore, the regulation of NF-κB plays an important role in colitis mediated colon carcinogenesis.

In both carcinogenic as well as inflammatory conditions, NF-κB is activated downstream of the inflammatory cytokines such as TNF-α and IL-1β. We presently studied the role of
several pro- and anti-inflammatory cytokines including IL-1β, IL-2, IFN-γ and TNF-α. We found the expression of pro-inflammatory cytokines (including IL-1β, IFN-γ and TNF-α) to be upregulated and the anti-inflammatory IL-2 to be suppressed in the DSS, DMH and DSS+DMH groups. These expressions were revived back towards normal by the co-administration of Celecoxib in these groups. This indicates that the chemopreventive action of celecoxib involves the downregulation of pro-inflammatory cytokines and NF-κB while upregulating the anti-inflammatory cytokines.

Mutations in the DNA repair enzymes lead to the build up of genetic mutations and hence contribute to carcinogenesis. Also, cell cycle checkpoints play a detrimental role in uncontrolled proliferation, a hallmark of cancer. The expression of the two DNA repair enzymes; MLH1 and MSH2, was studied and found to be suppressed in the inflammatory as well as carcinogenic conditions. The treatment with Celecoxib in these groups led to the upregulation of these levels. Cell cycle is regulated at early G1 phase by Cyclin D1/cdk4 complex while the G1 to S transition is mediated by CyclinE/cdk2 complex. The expression of both these complexes was elevated with the incorporation of DSS and DMH, alone as well as in combination. The co-treatment with Celecoxib reduced these levels towards normal. The negative regulators of cell cycle i.e. p53 and Rb, were also looked into for their role in colitis associated colon cancer and their levels were suppressed in inflammatory as well as carcinogenic conditions though celecoxib was able to correct these alterations to a significant extent.

Whether a cell will undergo oncogenic transformation is a delicate balance between cell proliferation and apoptosis. PCNA, a cell proliferation marker, was explored for its expression in the different treatment groups and it was found to be elevated in DSS, DMH and their combination group. The protein expressions of the pro-apoptotic Bad and Bax were downregulated while anti-apoptotic Bcl-2 expression was upregulated in these groups. Also, the expression of caspase-3, the ultimate executioner of apoptosis, was found to be decreased in the above mentioned groups while the administration of Celecoxib corrected these effects. Apoptosis was studied by fluorescent co-staining of the isolated colonocytes and in the paraffin embedded tissue sections by TUNEL assay and it was found that the extent of apoptosis was very low in the DSS and DMH treated animals while it was revived with the co-administration of Celecoxib in these groups.
Among the COX-independent effects of NSAIDs, the notable one is the regulation of the MAPK pathway and the PI3K pathway. Several components of these pathways were found to be upregulated with the administration of DSS and DMH but the treatment with Celecoxib lowered the levels of these agents. The protein expression of the transcription factors like Jak-3 and Stat-3 were also found to be elevated in the DSS, DMH and DSS+DMH groups while the co-administration of Celecoxib decreased these levels.

Suppression of angiogenesis is an important mechanism of action in the chemoprevention by NSAIDs. It plays an important role in the tumor progression to the advanced stages by forming new blood vessels from the pre-existing ones and helps in providing nutrients to the growing tumor. In the present study, we explored the role of VEGF and matrix metalloproteinases and found their levels to be raised in the inflammatory as well as carcinogenic milieu. The co-administration of Celecoxib with DSS and DMH decreased these levels towards normal, thereby showing that NSAIDs can be potent chemopreventive agents and their mechanism of action includes the suppression of angiogenesis.

The administration of Celecoxib modulates the various membrane properties such as decrease in membrane fluidity, increase in order parameter, decrease in lateral phase separation and increase in the membrane microviscosity. It was also found to up-regulate the intracellular Ca$^{2+}$ levels, which are highly critical in cancer as these lead to suppressed apoptosis, as seen with the DSS and DMH treatment.

To conclude, DSS-induced ulcerative colitis exaggerates the tumorigenic effects of DMH-induced colon carcinogenesis, thereby validating the deleterious role of inflammation in cancer as seen by various parameters, such as morphology, histopathology, study of cyclooxygenase enzymes, inflammatory cytokines, NF-kB, cell cycle check points, membrane properties, the oncogenic PI3K and the angiogenic pathway. Also, apoptosis was suppressed and proliferation enhanced with the co-administration of DSS with DMH. Celecoxib, a second generation COX-2 selective NSAID was found to significantly regress the effects of DSS and DMH, alone as well as in combination, and acted as a chemopreventive agent in ulcerative colitis mediated colon carcinoma in the present study.