Summary and Conclusion

Cancer chemoprevention is the use of natural, synthetic, or biological chemical agents to reverse, suppress, or prevent carcinogenic progression to invasive cancer. It is based on the concept of multistep carcinogenesis which describes a stepwise accumulation of alterations, both genotypic and phenotypic. Arresting one or several of the steps may impede or delay the development of cancer. The success of several recent clinical trials in preventing colon cancer in high-risk populations suggests that chemoprevention is a rational and appealing strategy.

In colon carcinogenesis an accumulation of genetic events within a single cell line leads to a progressively dysplastic cellular appearance, deregulated cell growth, and, finally, carcinoma. Colon cancer prevention has now focused on novel targeted therapies, such as nonsteroidal anti-inflammatory agents (NSAIDs, COX inhibitors). Advances in delaying the development of colon carcinoma have been shown in patients with FAP with NSAIDs treatment. Although, the use of COX-inhibitors in the primary prevention of sporadic colon cancer is being studied in several ongoing trials, their exact mechanisms of action are still largely not understood. The present work was envisaged to study the interplaying role of NSAIDs and colonic plasma membrane in combating the carcinogen (1,2-dimethylhydrazine) induced colon carcinogenesis in a rat model.

As evident from the results of the DMH dose standardization the higher dose group of DMH (i.e., 30 mg/kg b wt for 35 weeks) seemed to be the best dose (among 10, 20 and 30 mg/kg body wt groups) for establishing the rat model for colon carcinogenesis. These tumors were both polypoid (distinctly seen as abnormally raised polyps and a predominant type as seen in human right colon) and flat infiltrating tumors (predominant type as seen in human left colon). Histopathologically also these had striking similarity to those reported in the humans. Thus, the given dose of DMH and the given time schedule of the dose was considered adequate to establish the experimental model of colon carcinogenesis.
in rat. For further establishing the chemopreventive efficacy of NSAIDs at promoting and initiating stage of carcinogenesis, the animal groups comprising of Control, DMH, DMH + Aspirin, DMH + Celecoxib and DMH + Etoricoxib were carried out and dissections were done after 18 weeks (promotion stage) and 6 weeks (early stage) of the treatment period. Promoting stage of carcinogenesis was clearly evident after 18 weeks of DMH administration by both macroscopic and histopathological studies. Tumor incidence, tumor burden and tumor multiplicity were noted maximum in the DMH group while these values were minimally observed in the DMH + Celecoxib and DMH + Etoricoxib group. Histopathological examinations of epithelia show the occurrence of promoting stage of carcinogenesis in terms of great variety of carcinoma cases like signet ring cell carcinoma, invasive dysplastic ACFs associated with the lymphoid follicles and highly invasive carcinoma with transmural infiltrations. While in DMH + NSAIDs groups no malignancy noted, while the epithelia showed the occurrence of hyperplasia and early changes of aberrant crypt foci only.

When the treatment period was restricted upto 6 weeks an initiating stage of carcinogenesis was noted in which DMH group showed the occurrence of larger sized MPL (an initiating stage of cancer growth). However, with NSAIDs co-administration the size of the MPL was found to be markedly reduced and in DMH + Etoricoxib group, an occurrence of only minimum sized MPL was recorded. Histopathological results clearly indicated the characteristic neoplastic changes like hyperplasia, dysplasia and the adenoma formations at an early stage in the DMH group of animals with most of these malignant transformations being confined to the mucosal layer. Also, invasive crypts were found to be associated with the lymphoid follicles but no invasion of the deeper layer or of the muscularis mucosae were noted. These neoplastic transformations were found to be regressed where DMH was given along with the different NSAIDs in terms of lessening of mucosal inflammation, occurrence of ACF dysplasia and associated aggregates of lymphoid follicles. Despite extensive sampling there was no evidence of carcinoma in situ or Frank malignancy in any of the NSAIDs treated animals and therefore, indicated chemopreventive potential of these drugs during initiating stage of the colon cancer.

These observations therefore, clearly establish the onset of carcinogenic events as well as the regressing effects of NSAIDs. For studying in detail the role of membrane composition and physical state and evaluating the NSAIDs mediated...
Chemopreventive effects during an early stage of carcinogenesis, the experimental treatment duration was restricted to six weeks only.

Results of lipid composition studies showed the decrease in total lipids and cholesterol value in case of DMH treatments which was recovered in the NSAIDs groups. The phospholipid content was found to be increased with DMH treatments and similarly corrected in the NSAIDs groups. Among the NSAIDs tested, the values in the Etoricoxib + DMH reached close to the control values. Cholesterol: phospholipid (Chol:PL) ratio was found to be reduced remarkably with the DMH administration, however, in comparison to DMH group a significant improvement was observed with the NSAIDs treatment and the maximum recovery of the Chol:PL ratio was observed in DMH + Etoricoxib group. Similarly, in lipid biosynthesis studies radioactivity incorporated into cholesterol was found to be decreased with DMH as compared to the control. Co-administration of Aspirin and Celecoxib proved helpful in recovering the cholesterol biosynthesis in comparison to DMH group while DMH + Etoricoxib group recorded the highest increase in the same. Triglycerides and the fraction corresponding to the monoglycerides and diglycerides levels were increased in DMH group while NSAIDs treatment had reduced their biosynthesis. The lower levels were recorded in the DMH + Celecoxib and DMH + Etoricoxib groups. Phospholipid biosynthesis studies showed an enhanced level of S, PC and PE in the DMH group whereas with NSAIDs these fractions recorded lower concentrations in comparison to DMH group. Fraction corresponding to PI + PS content showed an increment in the DMH + NSAIDs groups with respect to the DMH group.

Levels of ganglioside sialic acid (GSA) were found to be elevated in DMH group as compared to the control whereas, DMH+NSAIDs treatment caused the substantial decrease in comparison to the DMH group only. Free fatty acid analysis using GLC showed that AA levels were decreased and palmitate levels were increased very significantly with NSAIDs co-administrations in comparison to the DMH group. Results as reflected by changes in the amount of lipids as well as its constituents, and the free fatty acids and the phospholipids biosynthesis pattern, clearly indicate that the cell membrane compositions are modified during DMH induced cancer transformation which are observed to revert back with the NSAIDs treatments.

Membrane dynamic studies were performed to assess the modulatory effects of altered lipid compositions on the membrane physical state parameters.
For this, membrane order parameter, lipid fluidity, membrane phase separations (membrane polarity) and membrane phase state were assessed using various membrane specific fluorescent probes. Membrane order as evaluated from the order parameter and lipid fluidity calculations showed an enhanced state of fluidity in DMH group which was corrected in the NSAIDs groups. Membrane polarity was assessed in terms of membrane phase separations using NBD-PE fluorescence quenching technique in liposomes, BBM vesicles and the colonocytes. Results showed that the DMH treatment has lesser lateral phase separation of membrane lipids whereas, NSAIDs treatment promotes the process indicating the presence of more ordered lipid domains (and less polar environment) in the bilayers of BBM vesicles, liposomes and plasma membranes of the colonic epithelial cells. Membrane phase state was evaluated by calculating the GP parameter using Laurdan as a membrane permeable fluorescent probe. Membrane phase state from the control values was recorded to be closer to the gel phase whereas for the DMH group values were noted to be closer to the liquid crystalline phase and in coxib groups (Celecoxib and Etoricoxib), once again a shift in GP values towards the gel phase was noted.

From the results obtained from membrane compositional and dynamics studies it seems that DMH by modulating membrane composition induces significant alterations at the membrane structural level and thereby alters its physical dynamics so as to make the conditions more favorable for the process of carcinogenesis. Conversely, NSAIDs may cause the membrane modulating effects by affecting the lipid phase separations and thereby causing the decrease in membrane polarity.

To evaluate the direct implication of altered membrane physical state and composition on the membrane functions, activity of sodium proton exchanger (NHE) - a major membrane ionic transport system in the colonocytes was assessed using acridine fluorescence quenching method. In the DMH administered group the NHE activity was found to be enhanced while the inhibition of this antiport activity was observed in all the NSAIDs + DMH treated animals. Of direct consequence of an altered NHE activity was the change in intracellular pH (pHi) which was found to be increased in DMH group while NSAIDs treatment showed the lowering of pHi. These observations, therefore strongly indicate that the NSAIDs exert their chemopreventive actions by regulating the pH, through an inhibition of the NHE activity.
Since increase in pH are known to induce apoptosis, further studies were carried out to reveal the underlying mechanisms of NSAIDs induced apoptosis. Various landmark molecular events leading to the apoptosis were analysed which included role of calcium homeostasis and activation of pro apoptotic signals like cytochrome-c release, PARP cleavage and activation of caspases along with analysing the status of anti-apoptotic proteins like bcl-2. Results of these studies showed that DMH induced carcinogenesis is folowed by an increased intracellular calcium level ([Ca^{2+}_i]) and also an inward calcium flux along with the inhibition of Ca^{2+} ATPase activity. These phenomena were found to promote the anti-apoptotic signals as seen by the increased bcl-2 protein expression. NSAIDs treatment on the other hand, proved beneficial in reversing the cancer promoting effects of DMH by initiating the apoptotic signals as evident by the increased release of cytochrome c and cleavage of PARP protein. Moreover, caspase 1 and 3 activities were also found to be higher in the NSAIDs treated groups further confirming the activation of apoptotic pathways. Additionally, morphological assessment and quantification of apoptosis were also done using fluorescent labelling of the colonocytes and TUNEL assay as well as DNA fragmentation analysis. These results established the occurrence of more number of apoptotic cells in NSAIDs treated groups while DMH only group showed negligible presence of apoptotic bodies, as also seen by more DNA fragmentation (ladder formation) in the NSAIDs treated groups.

It may be noted here that among the three NSAIDs tested, Etoricoxib (a highly selective COX-2 inhibitor) was found to be most effective in regressing the DMH induced carcinogenic effects as studied for all the present experiments. This provides a strong support to COX dependent basis of NSAIDs induced chemoprevention. To have better understanding of role of COX-2, experiments were also done to study the role of COX-2 activation during DMH induced carcinogenesis and NSAIDs chemoprevention along with analyzing the activation of iNOS and antioxidant status in the colonic mucosa as well as ROS generation in the colonocytes. The results from these experiments showed an increased expression of COX-2 in DMH group (both by Western immunoblot as well as immunohistochemical staining) which was found to be negligibly present in the Etoricoxib group. The lesser expression of COX-2 in the Etoricoxib group was found to be paralleled with the increased iNOS expression, nitrite level and ROS generation, all of which are known signals for inducing apoptosis. Further, All the enzymes of antioxidant defense system SOD, CAT, GR and GST as well as the non-enzymatic anti-oxidant GSH recorded a distinct decline in the DMH treated animals which were seen to be
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largely restored back when administered with the NSAIDs. The AOE and thiol levels showed a strong relation between the ROS generation and cellular apoptosis.

To conclude, the results of the present study clearly indicate that the alterations in the membrane physical state and composition do act as initiating events for inducing apoptosis and, therefore, can be considered as a strong molecular mechanism behind the chemopreventive actions of the NSAIDs in colon carcinogenesis. NSAIDs mediated apoptosis involves alteration in the NHE activity at a membrane level which is a crucial step for further initiating the various cytoplasmic events (such as pH, and calcium homeostasis) favorable for achieving the arrest of cellular proliferation or apoptosis. Further, the observed chemopreventive effects (as seen from gross anatomy, histopathology, membrane physical studies and other biochemical as well as the molecular physiological studies) were found to be more prominently associated with the selective COX-2 inhibitors like Celecoxib and Etoricoxib, as compared to the non specific COX-inhibitor Aspirin.