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Therapeutic Promise of Dabigatran Etexilate, an Oral Direct Thrombin Inhibitor in a Preclinical Model of Colon Carcinogenesis

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Abstract

Clinical observations suggest that there is activation of the coagulation system in patients of colon cancer. The resulting thrombin is implied in further exacerbation in the progression of colon cancer. We evaluated the effect of dabigatran etexilate (DE), an oral direct thrombin inhibitor in a preclinical model of 1, 2-Dimethylhydrazine (DMH) induced colon carcinogenesis in rats. DE reduced carcinogenesis induced gross morphological changes and colonic edema as compared to induced control. DE treatment significantly reduced levels of VEGF and ERK/MAPK and displayed a significant increase in colonic E-cadherin and reduction in N-cadherin, Twist and mTOR expression. Histopathologically, DE prevented the DMH induced adenocarcinomatous changes in the rat colon. Dual treatment of DE+5FU provided an additive effect as compared to the single treatment schedules of DE and 5FU, respectively. The preclinical study provides preliminary evidences on the promise of DE in treating DMH induced colon carcinogenesis in rats. The study portrays the effective role of DE treatment schedules both alone and in combination with 5FU in abating the parameters associated with progression of colon carcinogenesis expressing amelioration of factors mediating EMT, an important prelude to disease aggression and metastasis. A temporal association was drawn between administration of DE and improvement in colon cancer associated clinical end points. This investigation strongly suggests the role of thrombin in progression of carcinogen induced colon cancer and substantiates the role of direct thrombin inhibitors in treating colon cancer.

Keywords: Dabigatran etexilate; Thrombin; Oral direct thrombin inhibitor; 1, 2-Dimethylhydrazine; Colon cancer; Epithelial-mesenchymal transition; Additive effect

Abbreviations:

DE: Dabigatran Etexilate; DMH: 1, 2-Dimethylhydrazine; EMT: Epithelial-Mesenchymal transition; MET: Mesenchymal-Epithelial Transition; 5FU: 5-Fluorouracil; FOBT: Fecal Occult Blood Test; VEGF: Vascular Endothelial Growth Factor; ERK/MAPK: Extracellular Signal-Regulated Kinases; mTOR: Mammalian Target of Rapamycin; MAPK: Mitogen Activated Protein Kinases; PI3K/Akt/mTOR: Phosphatidylinositide 3-Kinases/Protein Kinase B/Mammalian Target of Rapamycin; HIF-1α: Hypoxia Inducible Factor-1α; ACF: Aberrant Crypt Foci

Introduction

In the 1860s, the French physician Armand Trousseau reported the occurrence of thrombotic disorders in cancer patients and concluded that spontaneous blood coagulation events are frequent in these individuals because of a 'special crisis in their blood' [1]. Since this publication, an important link between malignancy and hypercoagulable states was established [2,3]. The possibility of a relationship between clotting mechanisms and the development of metastasis was also postulated by Billroth in 1878, which described cancer cells within a thrombus and provided evidence of the spread of tumor cells by thromboemboli [4]. Additionally, thrombosis is often diagnosed as the first clinical manifestation of a tumor and the second leading cause of death of patients with cancer [3,5,6]. It is noteworthy that abnormalities in in vitro coagulation tests are found in more than 90% of patients with cancer, irrespective of their thrombotic status [7]. There is a prominent correlation between the incidence of thromboembolic events and a worse prognosis of neoplastic disease, supporting the idea that the activation of the blood coagulation system contributes to tumor aggressiveness and vice versa. The first-year survival rate of patients who are diagnosed with both cancer and venous thromboembolism (VTE) was 12%, in contrast with 36% observed in cancer patients without a diagnosis of thromboembolic events [8]. Patients with thrombosis-associated malignancies exhibited a higher mortality in the first 6 months of a thrombotic event than those individuals presenting with cancer without thrombosis or thrombosis without cancer [9]. Cohort studies and clinical trials approximate that10% of persons presenting with idiopathic VTE are subsequently diagnosed with cancer over 5 to 10 years, and diagnosis is established within the first year of presentation of deep vein thrombosis (DVT) in >75% of cases [10]. Acute VTE can be the first manifestation of an occult malignancy, and patients presenting with idiopathic VTE are more likely to have underlying cancer than those in whom a secondary cause of thrombosis is apparent [10]. Studies indicate that patients with malignant cancer and recurrent thrombosis have progressive metastatic cancer, worse prognoses, and poor survival rates [11]. These results are suggestive of the hemostatic system playing an important role in cancer pathogenesis.

Multiple components of the hemostatic system have been linked to cancer progression, particularly metastasis [12]. Previous studies in mice have explicitly shown that tumor cell–associated tissue factor (TF) [13-15], circulating prothrombin [13,16,17], and several downstream thrombin procoagulant targets (i.e., platelets, fibrinogen, factor XIII) [13,17-20] strongly promote tumor cell metastatic

potential. Harold Dvorak, 31 years ago precisely hypothesized that hemostatic factors contribute to the development of a supportive tumor stroma and tumor growth. It also stated that tumors pathologically replicate the wound-healing process and are ostensibly "wounds that do not heal" [21].

Colon cancer likely represents an important exception where hemostatic system components appear to drive aspects of cancer progression other than the formation of metastases. The probability of development of colon cancer in homozygous carriers of the prothrombotic factor V Leiden mutation is almost six times higher than non-carriers, signifying that thrombin generation is a significant element of colon tumorigenesis [22]. Consistently, studies in mice revealed that 50% decrease in circulating prothrombin significantly delayed the formation of colitis-associated colonic adenomas [23]. Furthermore, a substantial decrease in inflammation-driven adenoma formation was observed in fibrinogen-deficient mice; an effect coupled to fibrinogen-mediated engagement of the leukocyte integrin receptor αMβ2 [24]. These animal studies reveal an important role for prothrombin and fibrinogen in colonic adenoma formation.

Epithelial-to-mesenchymal transition (EMT) is a collection of events that allows the conversion of adherent epithelial cells, tightly bound to each other within an organized tissue, into independent fibroblastic cells possessing migratory properties and the ability to invade the extracellular matrix [25]. EMT is associated with repression of epithelial markers like E-cadherin and elevation of mesenchymal markers like N-cadherin [26] with increase in expression of transcription markers like Twist [27]. A study in 2011 [28] concluded that thrombin induced Twist directly upregulated N-cadherin through a HIF-1α-dependent pathway. The study showed for the first time that thrombin, acting through PAR-1, activates the HIF-1α signaling pathway, which in turn initiated Twist and N-cadherin expression and finally induced cell motility in colon cancer HCT-116 cells. These results suggested that in case of a constitutive imbalance in coagulants, tumors may acquire cell migration abilities. Evidence supports roles for the SMAD/STAT3 signaling pathway [29], the Ras-mitogen activated protein kinase/Snail/Slug [30,31], the NF-kB pathway [32-34] and micro RNAs [35-37] in the development of colon cancers via EMT transition. Thus, EMT appears to be closely involved in the pathogenesis of colon cancer.

Review of Literature

Thus, literature supports inter-dependence of coagulation system and colon cancer along with the involvement of EMT in pathogenesis of colon cancer. Despite these dependences, there are supportive but inconclusive evidences on the anti-neoplastic effect of warfarin, heparins and other antithrombotic agents, both experimental and clinical [38-46]. Direct thrombin inhibitors (DTIs) are a comparatively newer class of anticoagulants. Dabigatran etexilate (DE) has completed extensive clinical evaluation in various indications for anticoagulation therapy [47,48]. Moreover, recently a specific reversal agent, PraxbindTM (Idarucizumab), for reversal of excessive anticoagulation in an emergency has been approved in many countries [49]. A preclinical study by De Feo et al. [50] reported that oral administration of DE controlled both invasion and metastasis of malignant breast tumors.

It is well documented that the risk of colon cancer increases with prior history of Crohn's disease and Ulcerative colitis but vast majority of colon cancers do occur in absence of colitis [51].

Aim and Objectives

We utilized the carcinogen DMH induced rat model of colon cancer with an aim to evaluate the effect of an oral direct thrombin inhibitor on different stages of carcinogenesis. An important objective of the study was to evaluate the potential of oral direct thrombin inhibitor DE on various parameters associated with progression of colon carcinogenesis in the rat model of DMH induced colon cancer. We aimed to profile the molecular mechanism of action of DE with emphasis on EMT markers associated with the ERK/MAPK and PI3K/Akt/mTOR pathways knowing the fact that EMT has a pathologic role in colon cancer progression. The investigations compare the effect of DE treatment with that of 5FU, a drug which is the standard of care in colon cancer. It also explores the impact of dual DE and 5FU therapy on the clinical endpoints in the study.

Materials and Methods

Chemicals and kits

Dabigatran etexilate (DE) and 0.5% natrosol were provided by Boehringer Ingelheim GmbH, Germany. 1, 2-Dimethylhydrazine (DMH) was obtained from Sigma Aldrich, USA. 5-Fluorouracil (5FU) was obtained as a gift sample from Khandelwal Labs, Mumbai. DE was solubilized in 0.5% natrosol and given per orally insuspension. DMH was freshly prepared by solubilizing in saline and given interperitoneally (i.p.) as a clear solution. 5FU was solubilized in water (60-70°C) to a concentration of 1 mg/ml. The chemicals and reagents required for conducting FOBT were procured in house. The Elisa kits; Quantikine Elisa Rat VEGF Immunoassay kit and ERK/MAPK DuoSet IC Rat Elisa kit were obtainedfrom RandD systems, Minneapolis, MN, USA. For RT-PCR analysis, SV Total RNA isolation kit and GoTaq RT-PCR kit were purchased from Promega Corporation, WI, USA.

Animal experiments

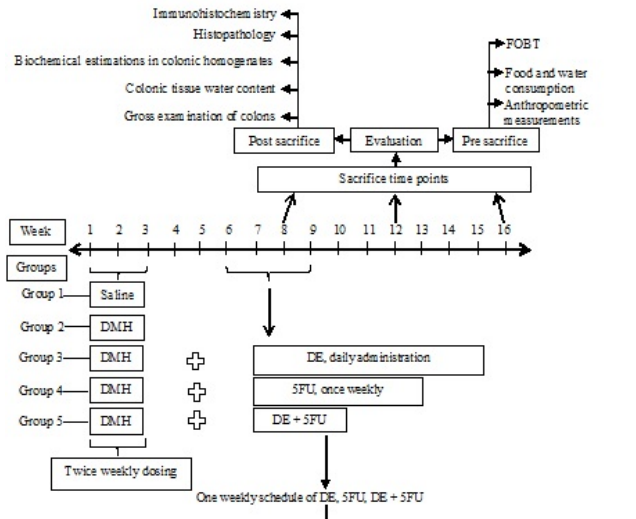
All animal studies were conducted after approval from the Institutional Animal Ethics Committee as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Government of India) guidelines (Approval No: CPCSEA-BCP/2014-02/04). Female Sprague Dawley (SD) rats that were 6-8 weeks old (weight 160-200 gm) were used for the present study and were obtained from Bharat Serums Ltd., Thane, India. Animals were housed in polypropylene cages, maintained under standard conditions (24 ± 2°C, 50 ± 10% relative humidity and 12 hours light/dark cycles) and provided with ad-libitum water and pelleted diet. The pellets were composed of 25.41% crude protein, 5.27% crude fibre, 10.87% moisture, 3.22% ether extract, 56.74% nitrogen free extract and 9.36% total ash.

Experimental design

Ninety female SD rats, 6-8 weeks old (weight 160-200 gms) were randomized into 5 groups composed of 18 rats/group. Injections of saline and DMH were administered i.p. twice a week for 3 weeks with at least 2 days between two injections. The study duration was of 16 weeks and followed an experimental design as shown in Figure 1.

Group 1 received normal saline. Remaining 72 rats were administered 30 mg/kg, i.p. of DMH and randomized into groups 2, 3, 4 and 5. Group 2 did not receive any treatment. Group 3 received DE (45 mg/kg, p.o, twice from Monday to Friday and 60 mg/kg once on Saturday and Sunday) from week 6-9 of the study duration. Group 4

received 5FU (50 mg/kg, i.p once a week on Wednesday) from week 6-9 of the study duration. Group 5 received dual treatment of DE+5FU (same dose and regimens) from week 6-9.



Groups	Treatment (mg/kg)	Time of administration	Weekly schedule from week 6-9						
			Mon	Tue	Wed	Thurs	Fri	Sat	Sun
Group 3	DE, p.o	Morning	45	45	45	45	45	60	60
		Evening	45	45	45	45	45	-	-
Group 4	5FU, i.p	Morning	-	-	50	-	-	-	-
Group 5	DE, p.o + 5FU, i.p	Morning	45	45	45 + 50	45	45	60	60
	DE, p.o	Evening	45	45	45	45	45	-	-

Figure 1: Experimental Design.

Assessment parameters

The pre-sacrifice parameters included measurement of body weight, food and water consumption and FOBT. Body weight of the rats and food and water consumption was documented on alternate days throughout the study duration. 500 ml of potable drinking water and 20 gm of feed/rat was refilled every second day. Fecal pellets of rats were collected at the end of week 8, 12 and 16 and FOBT was performed as per previously reported procedure.

The animals were sacrificed at the end of week 8, 12 and 16. Immediately after euthanasia, the colon was removed en bloc from the cecum to the anus and opened with scissors in the antimesenteric border and rinsed with ice-cold Phosphate buffered saline (PBS) solution, pH 7.4. Excised colon was emptied of contents, rinsed, and blot-dried on a light-duty tissue wipe. The colon was opened longitudinally and pinned out on a Petri dish to examine the gross colonic mucosa. The mucosal surface of the colon was inspected with a magnifying glass and photographs were clicked of the entire colon. Colonic edema was measured as difference between the wet and the dry weight of the colon. The wet weight of each segment was measured and allowed to air dry in a 37°C oven for 4 days after which its dry weight was recorded. The colons were stored at -80°C till further analyses. For all the resected colons, 3 cm of distal colon was cut, out of which 1 cm was stored in neutral buffered formalin (NBF) for histopathological and immunohistopathological analysis and the rest 2 cm were used for in vitro ELISA and RT-PCR analyses. One centimeter of distal colonic tissue was utilized for estimation of VEGF and ERK/MAPK by Elisa method. 5% rat colonic tissue homogenates were prepared in PBS, pH 7.4. All samples were analysed in duplicate. qRT-PCR was performed as a one-step process after sample RNA was extracted from the colonic tissues. Total RNA was isolated from the dissected colonic tissues (approximately 100 mg) using the SV total mRNA extraction kit (Promega Z3100, Madison, WI, USA) as per the manufacturer's instructions. One-step quantitative Real-Time RT-PCR was performed using One Step GoTaq® qPCR Master Mix (Promega, Madison, WI,USA) on StepOnePlus Real Time PCR (Applied Biosystems, CA, USA). The forward and reverse primers for E-cadherin, N-cadherin, Twist, mTOR and house-keeping geneglyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized commercially (Integrated DNA Technologies, Leuven Belgium) with following sequences.

E-cadherin-F:GTCACTGACACCAACGATAATCCT

E-cadherin-R:TTTCAGTGTGGTGATTACGACGTTA

N-cadherin-F: GGGTGGACGTCATTGTAGC

N-cadherin-R:CTGTTGGGGTCTGTCAGGAT

Twist-F: GACGACAGCCTGAGCAACA

Twist-R: CCACAGCCCGCAGACTTCTT

mTOR-F: GCAATGGGCACGAGTTTGTT

mTOR-R: AGTGTGTTCACCAGGCCAAA

GAPDH-F: CAAGGCTGAGAACGGAAGG

GAPDH-R: AGAGGGGGCAGAGATGATGA

The primers were reconstituted and diluted prior to addition in the experiment as per manufacturer's instructions. The real time PCR reaction mixture of 20 µl contained 10 µl of GoTaq qPCR master mix, 1.6 µl each of forward and reverse primer, 0.4 µl of GoScript RT mix, 2 µl of RNA template, 0.3 µl of CXR reference dye and nuclease free water to make up the volume to 20 µl. All experiments were carried out for over 40 cycles, with denaturation for 10 s at 95°C, annealing for 30 s at 60°C and extension for 30 s at 72°C. The mRNA levels were expressed as relative fold changes after normalization to GAPDH mRNA expression. Relative quantification of mRNA expression was calculated with the 2-ΔΔCT method. For histopathological examination, one centimeter of distal colonic sample showing visible disfigurations (defined reddish spots or areas, lesions, adhesions and overgrowth) was preserved in NBF for 24 hours, embedded in paraffin and cross sections of 5 µm were stained with hematoxylin and eosin. Histological sections were examined using a vector light microscope with a magnification of 100X and 400X. Immunohistochemistry was performed on paraffin-embedded, formalin-fixed colonic tumor tissue and normal sections which were cut three micrometer thick on silane or poy-L-lysine coated slide. Cut sections were transferred to three changes of xylene for 30 min followed by rehydration with decreasing grades of absolute alcohol, 95%, 70%, 50%. Then sections were washed under tap water for 20-30 min. Antigen was retrieved by heating the slides in citrate buffer pH 6 and microwaved in oven at 800 watt for 10 min, 420 watt for 10 min and 360 watt for 5 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 5 min. The slides were incubated for 10 min followed by incubation with primary antibody as per manufacturer's instruction for 30 min at room temp. Then washing was carried out in Tris buffer solution pH 7.4 for 10 min. Followed by incubation with super-enhancer for 10 min and again washed in Tris buffer solution pH 7.4

for 10 min. This was followed by incubation with poly Horseradish peroxidase (HRP) for 30 min and washing in Tris buffer solution pH 7.4 for 10 min. Later it was incubated with substrate 3,3'-Diaminobenzidine (DAB) and checked for color change, brown color appeared within 5-10 min, depending upon intensity of antigen-antibody reaction. Further it was washed in Tris buffer solution pH 7.4 for 10 min and transferred to tap water washing for 10-20 min. Then the slides were put into increasing grades of alcohol 50%, 70%, 95% and absolute alcohol and lastly transferred to three changes of xylene and mounted with Distyrene Plasticizer Xylene (DPX). Lastly, the slides were examined with a magnification of 100X and 400X.

Statistical analysis

One-way ANOVA with Tukey's post hoc test was performed using Graphpad Prism® Version 6.01 software, CA, USA. Data are presented as mean ± Standard Deviation (SD). Comparisons are made among Groups of the same week (*#†‡ considered significant, $P<0.05$). *Comparison of DMH group with saline control, #Comparison of DE, 5FU and DE+5FU group with DMH group, †Comparison of DE+5FU group with DE group, ‡Comparison of DE+5FU group with 5FU group.

Results and Discussion

There was no spontaneous mortality observed in this 16-week study. The rats in all groups maintained a healthy appearance. Bloody stools or episodes of diarrhoea were not detected throughout the 16-week study period.

Pre-sacrifice

Anthropometric parameters: Rats of group 1, 3, 4 and 5 showed consistent increase in the average body weight from 180 gm at week 0 to 250 gm at week 16. In group 2, DMH administration did not appreciably cause loss of body weight in rats during the initial 8 weeks of the study but the rats did display a significant weight reduction between 12th-16th week of the study as compared to group 1. Treatments to group 3 and 4 led to an increase in the body weight of the rats but was not found to be statistically significant. Group 5 rats exhibited significant increase in the body weight by the 16th week as compared to group 2.

Weight reduction in rats between weeks 12 to 16 in DMH induced group was probably caused by the cancer cachexia. Clinical correlation estimates that individuals with malignant tumors suffer from cachexia syndrome characterized by anorexia, anemia, loss of weight and tissue mass [52,53]. Groups 3, 4 and 5 showed consistency in weight throughout the study period and demonstrated that neither 5FU nor DE treated animals had any changes in body weight.

Food and water consumption: There was no significant difference in the consumption of food and water among all study groups. Each rat daily consumed average 16-20 gm of food and 30-35 ml of drinking water. None of the drugs used in the study led to any effects causing reduction in food and water intake.

FOBT: Occult blood was not found in the stools of rats across all study groups when performed at the end of week 8, 12 and 16.

Post-sacrifice

Gross necropsy: Group 1 displayed healthy colonic tissue throughout the study duration when observed at week 8, 12 and 16, with no visible signs of ulceration or lesions. The gross necropsy of group 2 captured the various stages and progression of colon cancer. After 8 weeks, the colon was pinkish in appearance, after 12 weeks reddish ulcers were visible and after 16 weeks a tumor was present on the distal colon. Colons from groups 3, 4 and 5 displayed visible reductions in redness and ulceration as compared to the Group 2 colons at the respective weeks (Figure 2).

The rats from the DMH-induced untreated cancer group exhibited tumor localization mainly in the distal colon, and the observed tumors were mainly exophytic, which is in good agreement with reported histogenesis of human colon cancer [54,55]. Visual inspection of the colons at the end of weeks 8, 12 and 16 showed the progression of carcinogenesis of DMH induced group grading from redness, lesions and adenocarcinoma, respectively. The adenocarcinoma displayed features similar to human carcinomas, including a thickened red swollen mass of cells growing outwards beyond the epithelium from where it seemed to originate. Exophytic tumors were predominantly localized in distal colon, whereas endophytic ones were localized in proximal colon. It was found that DE and 5FU mainly affected the growth of exophytic tumors (adenomas and hyperplastic polyps). As compared to the positive control, the DE-treated group showed less gross visual changes and tumorous growth was not observed. Rats treatment with 5FU or the combination of 5FU and DE presented with lesser gross colonic abnormalities.

Colonic edema: Colonic edema was absent in group 1, with a weight of 0.05 gm at week 8 remaining unchanged till week 16. Colonic edema in Group 2 increased 5-6 fold over the duration of the study and at the 16th weeks the weight was 6-fold greater than that seen in group 1. Significant increase in the colonic water content of group 2, indicative of colonic edema was observed after week 8 which consistently increased till the 16th week of the study. Colonic edema of groups 3, 4, 5 was found significantly less as compared to group 2 at the respective time points. The rats of the dual treatment Group 5 showed notable reduction in colonic edema as compared to individual treatments schedules of DE and 5FU.

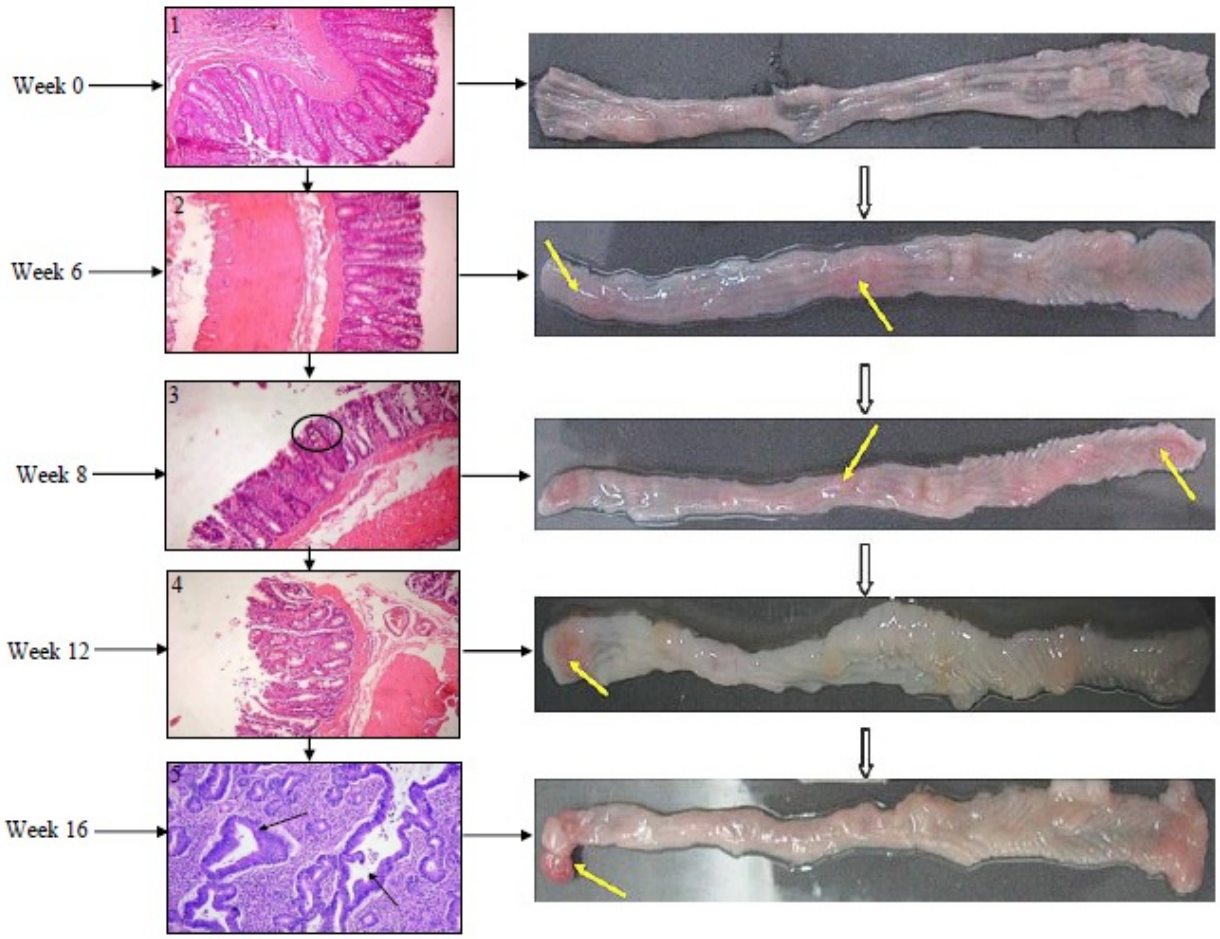


Figure 2: Histopathology and corresponding gross necropsy of the rat colon,1: Normal rat colon with well-defined histology and grossly it appears a normal healthy colon; 2: 6th week – The mucosa showed mild to moderate degree inflammatory changes and mild degree desquamation of mucosal epithelium while gross examination showed an overall pinkish appearance of the colon; 3: 8th week – Minimal no of ACF with mild inflammatory changes in the mucosa, mild degree desquamation of epithelium and gross morphology displayed ulceration with reddish spots in middle colon; 4: 12th week – Moderately multifocal ACF with mild to moderate inflammatory changes, minimal disintegration of crypts and mild to moderate degree desquamation of epithelium. Grossly, prominent edema with reddish spots in distal colon was seen; 5: 16th week – Mucosa showed extensive, complex epithelial growth pattern with moderate amount of intervening stroma. The proliferating cells formed vilo grandular structures. Budding and branching of glands formed papillary structures. Invasion of submucosa was seen. The nuclei were dark, nucleoli were prominent. Multifocal benign squamous differentiation was seen. In gross necropsy, an adenocarcinoma in distal colon can be distinctly seen.

A segmental distribution of colon wall thickening that is distal to a large fungating mass can also be present in approximately 10% of patients with colon cancer, and this pathologically corresponds to edema or colitis [56]. In fact, reports suggest that edematous bowel wall can obstruct the visualization of any lead mass of tumors [57,58]. We measured colonic water content as an indirect estimate for edema and found that it was significantly higher in DMH treated group indicating colonic edema induced by the carcinogen. On DE treatment, colonic edema was significantly reduced thus signifying that DE can control the colonic inflammation associated with colon cancer.

VEGF: An average VEGF level ranged from 80 to 116 pg/ml in the saline control group from week 8 to 16 (Figure 3). This range was significantly elevated in DMH induced group 2, which were analysed with more than 235 pg/ml of VEGF at week 8 that rose to 800 pg/ml at week 16. Across all respective time points, treatment groups 3, 4 and 5 displayed significantly less VEGF levels as compared group 2. Group 5 of DE+5FU significantly reduced VEGF levels in comparison to individual treatment groups of DE and 5FU at weeks 8, 12 and 16.

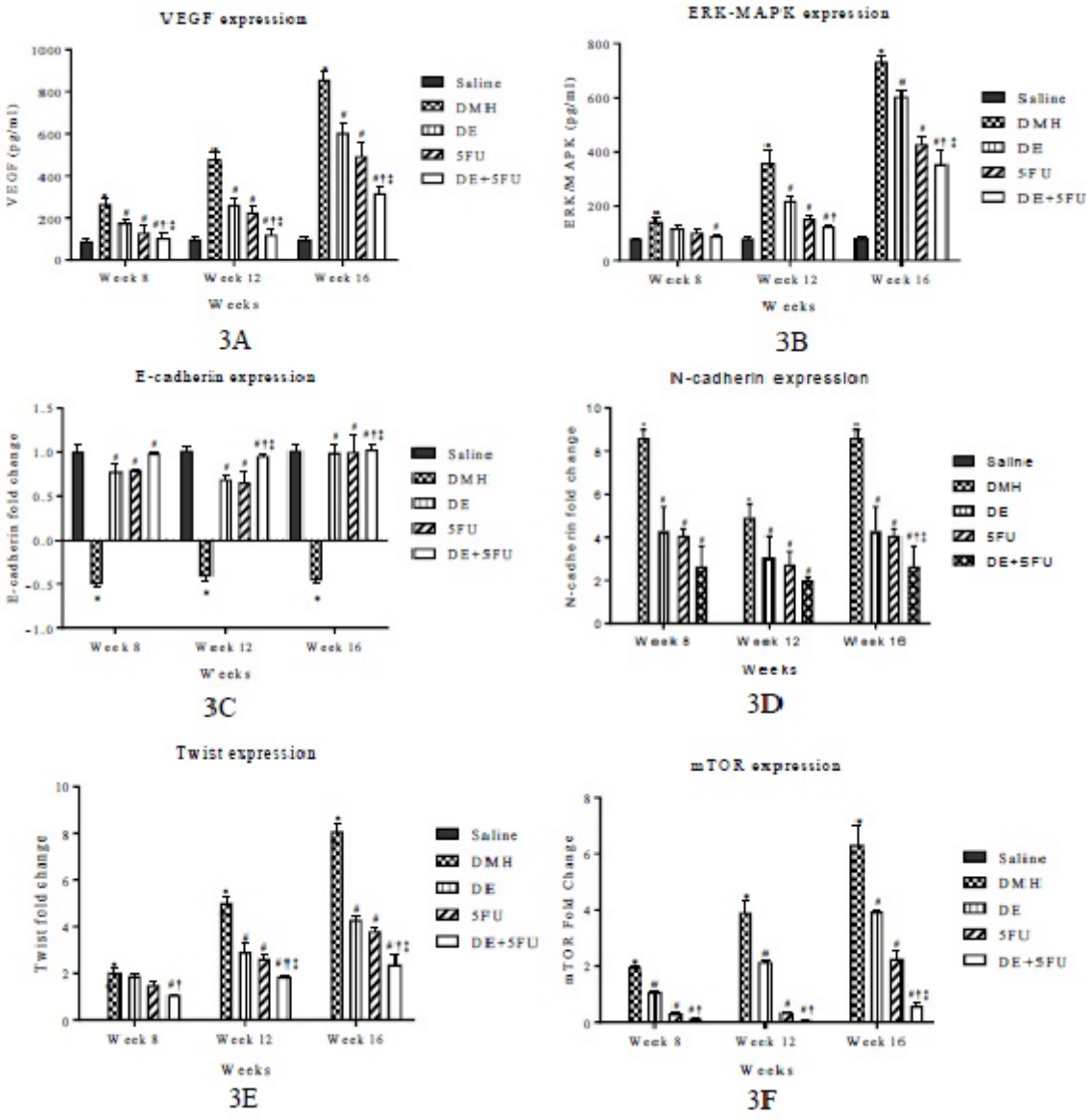


Figure 3: Biochemical estimations in colonic homogenates. Comparisons are made among Groups of the same week (*#†‡ considered significant, P<0.05). *Comparison of DMH group with saline control, #Comparison of DE, 5FU and DE+5FU group with DMH group, †Comparison of DE+5FU group with DE group, ‡Comparison of DE+5FU group with 5FU group. Fold change (FC) calculated after normalization to GAPDH mRNA expression. A: Measurement of VEGF (pg/ml) in colonic homogenates; B: Measurement of ERK/MAPK (pg/ml) in colonic homogenates; C: E-cadherin expression in terms of FC; D: N-cadherin expression in terms of FC; E: Twist expression in terms of FC; F: mTOR expression in terms of FC.

Tumor growth requires new blood vessel formation i.e angiogenesis. Evidence from preclinical and clinical studies indicates that VEGF is the predominant angiogenic factor in colon cancer [59,60]. VEGF expression is associated with advanced tumor progression and a poor prognosis in colon cancer. Activation of the VEGF/VEGF receptor axis triggers multiple signaling networks that result in endothelial cell survival, mitogenesis, migration and differentiation. VEGF also mediates vessel permeability and has been associated with malignant effusions [61]. Indeed, VEGF is expressed in approximately 50% of colon cancers with minimal to no expression in normal colonic mucosa and adenomas [62]. In the current study, VEGF levels upon treatment with DE showed significant reduction as compared to the DMH induced group which indicates a favorable role of DE in controlling the formation of newer blood vessels and thus abating the metastasis of colon cancer.

Estimation of ERK/MAPK

Significantly higher average ERK/MAPK levels were recorded in group 2 rats as compared to other groups (Figure 2). In group 2, there

was an exponential increase in ERK/MAPK from 1.6 to 3 to 8 fold rise from week 8 to 12 to 16, respectively. DE treatment prevented the rise in ERK/MAPK levels significantly compared to the untreated DMH administered group 2. Across all respective time points, groups 3, 4 and 5 displayed significantly less ERK/MAPK levels as compared to respective weeks of group 2. Group 3, 4 and 5 exhibited comparable decrease in ERK/MAPK levels at week 8. At week 12, Group 5 showed more pronounced reduction in ERK/MAPK levels in contrast with Group 3. While at week 16, the dual DE+5FU treated rats expressed notably lower levels of this marker as compared to the rats treated with either DE or 5-FU of Groups 3 and 4, respectively.

The ERK pathway is one of the best studied MAPK pathways and is deregulated in one third of human cancers including colon cancer [63]. We found that ERK/MAPK levels were raised in the DMH induced group and DE treatment significantly reduced the levels. Thus, DE's role in controlling unusual forms of intra and inter-communication like ERK/MAPK leading to unusual cell proliferation can be indicated.

Analysis of EMT associated protein mRNA expression by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

E-cadherin: The colonic E-cadherin expression was found to be significantly downregulated in group 2 as compared to saline control normal group at all the 3 weeks' time points (Figure 3). Groups 3, 4 and 5 displayed significant increase in the expression of E-cadherin as compared to Group 2. By week 16, E-cadherin expression was found to be comparable with saline control in all the treatment groups 3, 4 and 5. At week 8, DE+5FU showed comparable increase in E-cadherin expression as compared to DE and 5FU treatments, per se. While at week 12 and 16, the dual treatment was significantly more effective in raising E-cadherin expression as compared to individual treatments.

N-cadherin: There was minor detection of N-cadherin in only 2 samples of Group 1 at all three time points. The colonic N-cadherin expression was significantly upregulated in Group 2 across all the time points as compared to saline control normal group. Treatment with DE, 5-FU and the combination significantly reduced its expression as compared to group 2 (Figure 3). Group 5 rats given the dual treatment exhibited the lowest N-cadherin expression at week 16 while the effect was comparable to individual treatment groups of 3 and 4 at week 8 and 12.

Twist: Expression of Twist was not detected in group 1 at the end of weeks 8, 12 and 16. In group 2 it was significantly elevated as compared to group 1 at all three time points (Figure 3). Although group 5 showed significant reductions in Twist expression compared to group 2 at week 8, individual treatments did not show any significant reduction. At weeks 12 and 16, treatment groups 3, 4 and 5 presented with significantly less expression of Twist as compared to respective expression recorded in group 2. Dual treatment of DE+5FU notably reduced Twist expression at all weeks' 8, 12 and 16 in comparison to DE and 5FU individual treatments.

The E-cadherin-catenin complex provides cell-cell adhesion. In order for the carcinoma to metastasize, cancer cells must let go off their hold of neighboring cells in the primary tumor which is quite why E-cadherin is reduced during EMT [64]. N-cadherin is the mesenchymal marker and its detection is associated with poor prognosis and clinical outcome of colon cancer [65]. Twist is a transcription factor that regulates EMT in various cancers including colon cancer [66]. In our study, we found that in carcinogen induced group, mRNA expression of E-cadherin was downregulated and of N-cadherin and Twist was upregulated signifying EMT. After DE treatment, the expression of E-cadherin was increased and N-cadherin and twist expression was reduced indicating restoration of the normal colonic epithelium.

mTOR: Expression of mTOR in group 1 was not detected at the end of week 8, 12 and 16. Group 2 showed significant rise in the gene expression at week 8, 12 and 16 when compared to group 1 (Figure 3). All treatment groups 3, 4 and 5 exhibited significant reduction in mTOR expression in comparison with respective gene expression found in group 2. mTOR expression was significantly less in group 5 as compared to group 3 at week 8 and 12 whereas at week 16, it was exhibited marked declined in expression as compared to both individual treatment groups 3 and 4.

A likely candidate of biomarkers for colon carcinomas is the mTOR, a Serine/Threonine protein kinase which plays a key role in regulating important cellular functions, including proliferation [67], growth [68], survival [69], mobility and angiogenesis [70]. In several non-colon tumors, activation of the mTOR pathway and overexpression correlate with more aggressive clinical courses and has been reported to be useful target therapy [71-73]. mTOR is also reported to be actively involved in EMT as silencing its two complexes mTORC1 and mTORC2 lead to a array of biochemical, morphological and functional changes which are characteristic of mesenchymal Epithelial transition (MET) thus beneficial in controlling the carcinogenesis [74]. Similarly, ex vivo immunohistochemical studies on human colon adenomas and cancers confirmed that mTORC1 signaling occurs as an early event in the process of tumorigenesis and participates in the progression of normal cells to a neoplastic phenotype [75]. In this study, we found that DE significantly reduced mTOR expression which was upregulated in the carcinogen induced group.

Histopathological examination

Group 1 displayed normal histology throughout the study duration with all colonic layers well defined and intact. Graded stages of colon carcinogenesis from ACF at week 8 to adenoma in week 12 and adenocarcinoma in week 16 were observed in group 2 (Figure 2). The gross necropsy of colonic tissue of all groups correlated well with the histopathological findings (Figure 4). Notable reduction in histopathological changes induced by DMH was observed in colons of group 3, 4 and 5.

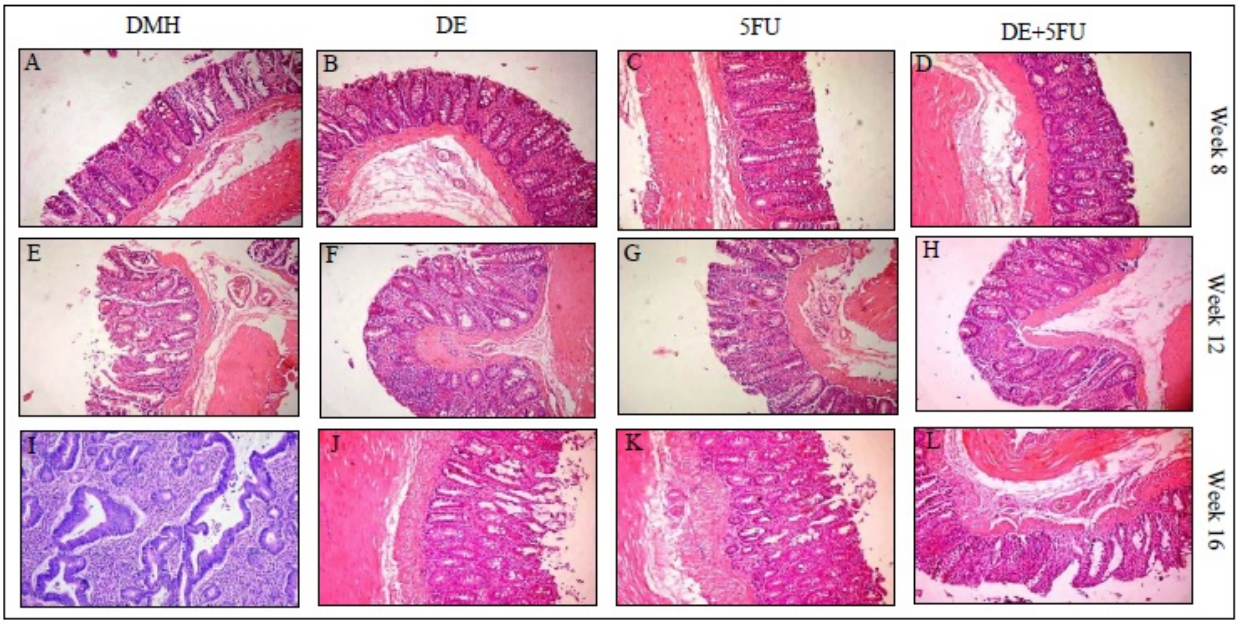


Figure 4: Histopathological examination of colon at week 8, 12 and 16. A: DMH induced: Minimal no of ACF with mild inflammatory changes in the mucosa, mild degree desquamation of epithelium; B: DE treated – Occassional ACF with moderate degree inflammatory changes in the mucosa, mild degree desquamation of epithelium; C: 5FU treated – Occassional ACF with moderate degree inflammatory changes in the mucosa, mild degree desquamation of epithelium; D: DE+5FU treated – Mild degree inflammatory changes in the mucosa, mild degree desquamation of epithelium; E: DMH induced: Moderately multifocal ACF with mild to moderate inflammatory changes, minimal disintegration of crypts and mild to moderate degree desquamation of epithelium; F: DE treated – Mildly multifocal ACF, mild inflammatory reaction in the mucosa and mild to moderate degree desquamation of epithelium; G: 5FU treated – Minimally multifocal ACF, mild to moderate inflammatory reaction in the mucosa and mild to moderate degree desquamation of epithelium; H: DE+5FU treated – Mild inflammatory reaction was observed. The colon crypts showed minimal disintegration and desquamation of epithelial lining of moderate severity. I: DMH induced: Mucosa showed extensive, complex epithelial growth pattern with moderate amount of intervening stroma. The proliferating cells formed vilo-grandular structures. Budding and branching of glands formed papillary structures. Invasion of submucosa was seen. The nuclei were dark, nucleoli were prominent. Multifocal benign squamous differentiation was seen; J: DE treated – Moderate degree inflammatory changes in the mucosa and minimal desquamation of epithelium; K: 5FU treated – Mild degree disintegration of colon crypts. Moderate to severe inflammatory changes in the mucosa and moderate degree desquamation of epithelium; L: DE+5FU treated – Mild degree inflammatory changes in the mucosa and minimal desquamation of epithelium.

In the present study, the histopathological findings in the colons of the DMH induced rats of group 2 were in agreement with human colon cancer, covering all the stages of carcinogenesis from ACF to adenomas to carcinomas. In alignment with prior reported studies [76,77] ACF were observed in the DMH group at week 6-8 which progressed to adenomas around week 12, and further advanced to adenocarcinomas. These adenocarcinomas depicted the classical colon cancer picture of submucosal invasion and accompanying fibroblastic stromal reaction. All treatment groups reduced the histological aberrations to a considerable extent giving a rational suggestion that DE could be beneficial in human colon cancer considering the multiple resemblances between DMH model with human colon cancer across varied aspects of molecular and histological mechanisms.

Immunohistochemistry based estimations of the colon

E-cadherin: E-cadherin % positivity was found to reduce with time in group 2 from 50% to 15% as compared to saline control which displayed 85-90% E-cadherin positivity. The colons of groups 3, 4, 5 displayed consistent increase in E-cadherin % positivity at week 8, 12 and 16 (Figure 5). E-cadherin positivity in DE group increased from 50% to 80% from week 8 to 16. Both 5FU and combination group showed increased E-cadherin presence from 80% to 95% from week 8 to 16.

N-cadherin: The colons from rats of normal control group tested negative for N-cadherin % positivity. In group 2, it was found to 70-75 at week 8 and 75-80 at both week 12 and 16. Groups 3 and 4 showed reduction in N-Catherin positivity as compared to group at all the time points (Figure 6). N-cadherin in DE treated group reduced from 50% at week 8 to 10% at week 16. In 5FU group, N-cadherin expression declined from 10% to 5% from week 8 to 16. In group 5 at week 12, 5% tissue tested positive for N-cadherin and at week 16, no expression of N-cadherin was detected.

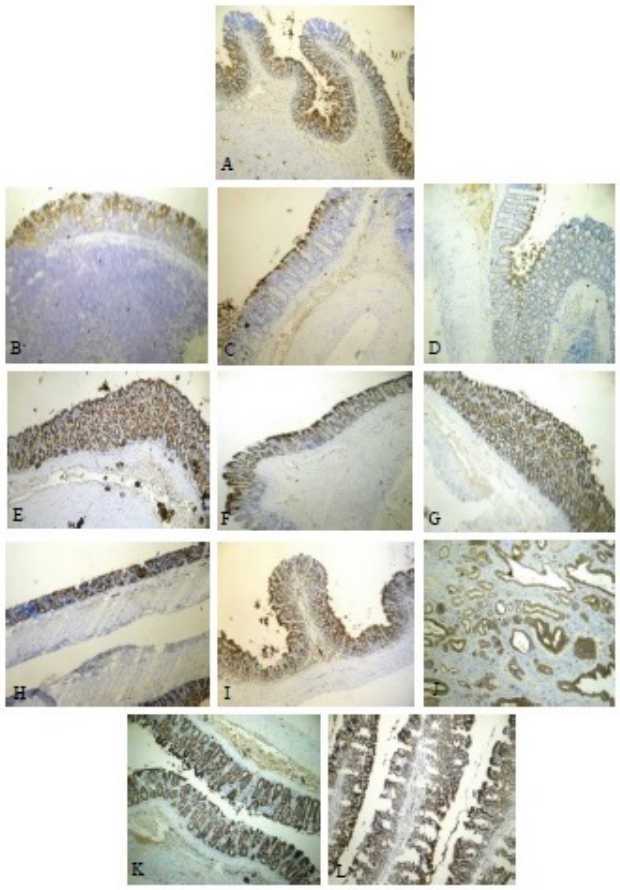


Figure 5: Immunohistochemistry of E-cadherin expression. Immunohistochemistry for E-cadherin expression in normal colon (A); DMH induced colon samples (B-D); DE treated colon samples (E-G); 5FU treated colon samples (H-J) and DE+5FU treated colon samples (K-L). Magnification 100X.

In our study, we limited our immunohistochemical analysis to major EMT parameters like E-cadherin and N-cadherin. In DMH induced group 2, progressive decrease in detection of E-cadherin and a gradual increase in N-cadherin expression was symbolic of EMT. In the treatment groups treated with DE, 5FU and DE+5FU, the pattern showed reversal wherein there was steady rise in E-cadherin and reduction in N-cadherin expression thus indicative of re-establishment of normal colonic epithelium and controlling the colon cancer induced by DMH.

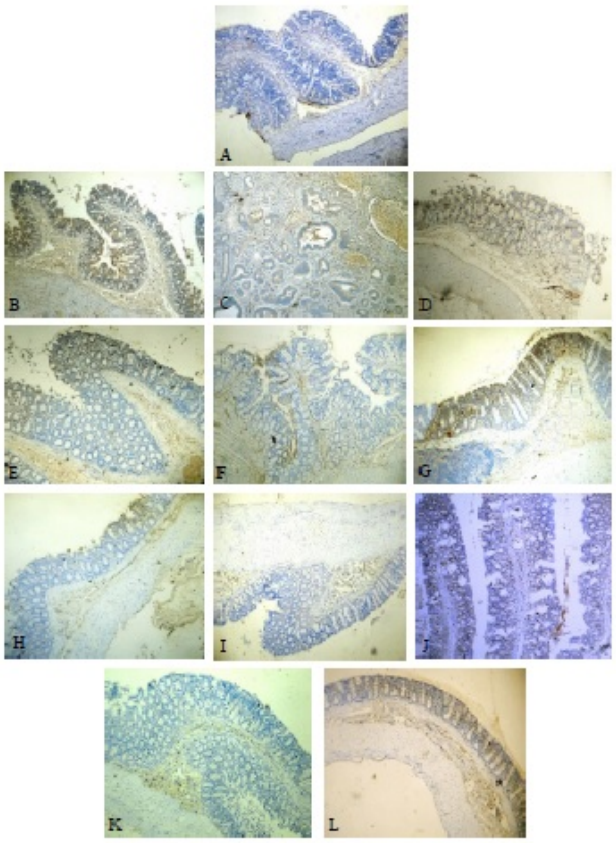


Figure 6: Immunohistochemistry of N-cadherin expression. Immunohistochemistry for N-cadherin expression in Normal colon (A); DMH induced colon samples (B-D); DE treated colon samples (E-G); 5FU treated colon samples (H-J); DE+5FU treated colon samples (K-L). Magnification 100X.

Conclusion

DE is an oral, reversible thrombin inhibitor that has been previously shown to reduce the invasive behavior of breast cancer cells in vitro and reduce tumorigenicity and metastatic behavior in vivo [50]. Depending on the prognostic significance of blood coagulation parameters and histopathological observations of an earlier conducted pilot study in our laboratory, we initiated the DE treatment at week 6 i.e at the stage of aberrant crypt foci (ACF) which are documented biomarkers for colon cancer [76,77].

Reiterating the Trousseau syndrome of link between cancer and coagulation, thrombin via PAR-1 is believed to play a critical role in embryonic and adult vascular development and also in EMT by virtue of which tumor cells convert themselves from low to high grade for distant metastasis [78,79]. Also thrombin-induced HIF-1α protein occurs through PAR-1 activation and it is regulated by MAPK and PI3K/Akt/mTOR pathway is a previously reported observation. The same study also indicated that Twist gene is critical for HIF-1α-mediated EMT and metastasis [80]. With the cognizance of the above reports and facts, we assessed the effects of DE on the EMT in a DMH induced colon cancer model in rats.

One of the important observations of our study was that both DE, or its co-administration with 5FU did not cause or worsen thromboembolic complications in our model, particularly since chemotherapy is a reported risk factor for thrombotic complications in cancer patients. DE treatment was strategically initiated at week 6 coinciding with the presence of ACF and progression through the early stages of adenomas detected later. Also, EMT markers began to surge at early stages of colon carcinogenesis (week 8 in our study) when an E-cadherin and N-cadherin imbalance was observed. With DE treatment, ACF development and the EMT was reversed significantly. ACF can be clinically diagnosed with routine colonoscopy. Thus, if an antithrombotic like DE is introduced at an early stage, it may delay the onset of colon cancer. In addition, the incidence of ACF occurred with EMT, thus DE initiation at this stage may help in reversal of EMT and provide better control over advancement of colon cancer. Nevertheless, due to its antithrombotic activity and inhibitory effect on thrombin, which is a contributory factor in metastatic progression, DE maybe a worthwhile complementary treatment to the existing chemotherapy for colon cancer.

Another noteworthy observation of this study was the beneficial effect of the combination group DE+5FU in controlling the colon cancer where across all parameters it can be observed that the combination group towards week 16 conferred an additive cancer control as compared to monotherapy of DE and 5FU.

Our results thus support the hypothesis that DE by virtue of its direct thrombin inhibitory action can be of help in inhibiting the ERK/MAPK and mTOR pathways leading to VEGF reduction and resulting in reversal of EMT.

We conclude that dabigatran was effective in reducing cancer progression in DMH-induced colon cancer in rats by inhibiting thrombin wherein it abated ACF which is an early diagnostic marker of colon cancer and ameliorated eventual EMT. This reiterates that thrombin plays a role in colon cancer progression. Co-treatment of DE with the standard drug 5FU can potentiate the chemotherapeutic outcome of 5FU and thus offer additive inhibition on progression of colon cancer. The study demonstrates for the first time, the therapeutic effectiveness of DE alone or in combination with 5FU in a preclinical model of colon cancer in rats. Further preclinical studies to assess and confirm the effectiveness and safety of DE in colon cancer are needed to lay a foundation for clinical studies in future.

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Conflict of Interest and Financial Disclosure

None.

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