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Non steroidal anti-inflammatory drugs modulate the physicochemical properties of plasma membrane in experimental colorectal cancer: a fluorescence spectroscopic study

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Abstract According to "fluid-mosaic model," plasma membrane is a bilayer constituted by phospholipids which regulates the various cellular activities governed by many proteins and enzymes. Any chemical, biochemical, or physical factor has to interact with the bilayer in order to regulate the cellular metabolism where various physicochemical properties of membrane, i.e., polarization, fluidity, electrostatic potential, and phase state may get affected. In this study, we have observed the in vivo effects of a procarcinogen 1,2-dimethylhydrazine dihydrochloride (DMH) and the two non steroidal anti-inflammatory drugs (NSAIDs); sulindac and celecoxib on various properties of the plasma membrane of colonocytes, i.e., electric potential, fluidity, anisotropy, microviscosity, lateral diffusion, and phase state in the experimentally induced colorectal cancer. A number of fluorescence probes were utilized like membrane fluidity and anisotropy by 1,6-diphenyl-1,3,5-hexatriene, membrane microviscosity by Pyrene, membrane electric potential by merocyanine 540, lateral diffusion by *N*-NBD-PE, and phase state by Laurdan. It is observed that membrane phospholipids are less densely packed and therefore, the membrane is more fluid in case of carcinogenesis produced by DMH than control. But NSAIDs are effective in reverting back the membrane toward normal state when co-administered with DMH. The membrane becomes less fluid, composed of low electric potential phospholipids whose lateral diffusion is being prohibited and the membrane stays mostly in relative gel phase. It may be stated that sulindac and celecoxib, the two NSAIDs may exert their anti-neoplastic role in colorectal cancer via modifying the physicochemical properties of the membranes.

Keywords Colon carcinogenesis · Fluorescence spectroscopy · Membrane fluidity · Non steroidal anti-inflammatory drugs

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Introduction

Mammalian cellular bilayers are constituted mainly by the phospholipids and the sphingolipids which represent the environment where many functional proteins and enzymes are dissolved. Depending upon the length and degree of unsaturation of the acyl chain, phospholipids could be in the gel or in the liquid-crystalline phase at a particular physiological temperature range. Phospholipids and sphingolipids-like ceramide show a large compositional heterogeneity and non-ideal miscibility in membranes giving rise to the possibility of domain co-existence [1]. These lipid domains or "rafts" are thought to have important roles in cellular phenomenon like signal transduction, and intracellular sorting of membrane proteins and lipids [2]. Behavior of membrane proteins can be strongly affected by the variations in the lipid composition and physical state [3]. Several physiological processes such as the cellular proliferation and differentiation are also involved in the modification of the composition and physical properties of the phospholipids in the natural membranes. Any variation in phospholipid structures or phase state is believed to affect the activity of membrane associated proteins [4]. The physiological characteristics of membrane have become important to diagnose and explain pathological mechanisms in case of chronic inflammations, carcinogenesis, and cardiovascular diseases and even aging [5].

Recently, non steroidal anti-inflammatory drugs (NSAIDs) have generated considerable interest as a promising chemopreventive agents for various cancers, e.g., colon, gastric, pulmonary, ovarian, and prostate [6]. It had been observed that NSAIDs act as anticancer drugs by inhibiting Cyclooxygenase (COX), a rate limiting enzyme in prostaglandin biosynthesis. There are two isoforms of the enzyme: COX-1 and COX-2 which are differentially inhibited by the NSAIDs. The anti-neoplastic activity in colorectal and other cancers of these drugs is shown to be mediated via inhibiting COX-2 enzyme which is particularly over-expressed in the neoplasm [7]. Like other drugs the function of NSAIDs is influenced by their lipid affinity as they can interact with either hydrophilic head groups or hydrophobic aliphatic tails depending on their hydrolipophilic characteristics. As the activities of different membrane bound proteins, enzymes, and signal transducer molecules are dependent on the cellular membrane characteristics like fluidity and local curvature, it can be said that the chemopreventive effects of NSAIDs may also be dependent on their ability to interact with the lipids. Interaction of NSAIDs with the membrane lipids can be indicated by their respective high membrane partition coefficients [8].

So far, however, the relationship of the anti-carcinogenic effects of the NSAIDs and the membrane physicochemical properties such as fluidity change had not been demonstrated, particularly in chemically induced colon cancer in animal model. This 1,2-dimethylhydrazine dihydrochloride (DMH) treated animal model is acceptable throughout the literature for chemically induced colon cancer studies and represents histopathological, cellular, and molecular similarities with the human cases [9–12]. In this study, therefore, we have examined the chemopreventive effects of two NSAIDs (sulindac, a non-specific COX-2 inhibitor and celecoxib, a selective COX-2 inhibitor) on the plasma membrane fluidity and other characteristics in the isolated colonocytes. The colonic cells depict the same characteristics of the cells in the tissue architecture if the isolation procedure involves less stress to the plasma membrane and similar physiological conditions as in vivo.

Materials and methods

Chemicals

1,2-dimethylhydrazine dihydrochloride, 1,6-diphenyl-1,3,5-hexatriene (DPH), Pyrene, merocyanine 540 (5-[3-sulfopropyl-2(3H)-benzoxazolylidine)-2-butenylidene]-1, 2-dibutyl-2-thiobarbituric acid), *N*-NBD-PE (L-α-Phosphatidylethanolamine dipalmitoyl-7-nitrobenzene-2-oxa-1,3-diazol-4-yl), Laurdan (6-dodecanoyl-2-dimethylaminonaphtalene), and sulindac were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS, Calcium and Magnesium free) and Morpholino propane sulfonic acid were from Himedia, Mumbai, India. Primary antibodies against COX-2 and anti-mouse β-actin were purchased from Santa Cruz Biotechnology (CA, USA). Alkaline phosphatase-conjugated secondary antibodies, Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies and BCIP–NBT were purchased from Genei (Bangalore, India). celecoxib was a generous gift from Ranbaxy, Gurgaon, India. All other chemicals and reagents used in this study were of analytical grade and procured from the reputed Indian manufacturers.

Animal procurement

Male Sprague–Dawley rats of body weight between 150 and 200 g were obtained from the inbred population of the Central Animal House, Panjab University, Chandigarh. These were acclimatized to the control diet (rodent chow) and water ad libitum for at least 1 week. Animals were maintained as per the principles and guidelines of the Ethics Committee of Animal Care of Panjab University in accordance with the Indian National Law on animal care and use. The animals were housed three per cage in polypropylene cages with a wire mesh top and a hygienic bed of husk (regularly changed) in a well ventilated animal room till the end of the experimental period. The animals were also maintained at the ambient temperature and humidity, and under a 12 h photoperiod of light and darkness, respectively.

Treatment schedule

Animals were assorted into the following groups:

Control group, vehicle treated: Animals were administered the vehicle (1 mM EDTA–saline subcutaneously (s.c.) in weekly injection and 0.5% carboxymethyl cellulose sodium salt per oral (p.o.) daily.

1,2-dimethylhydrazine dihydrochloride group: Animals were administered with DMH weekly at a dose of 30 mg/kg body weight (s.c.). The dose of DMH has been established in our laboratory earlier [9, 10]. DMH was freshly prepared in 1 mM EDTA–saline, pH adjusted to 7.0 using dilute NaOH solution.

DMH + sulindac group: sulindac was given daily per oral (p.o.) within its therapeutic anti-inflammatory dose (ED_{50} for rats, 20 mg/kg body weight) to the animals along with the weekly administration of 30 mg/kg body weight of DMH [11, 12].

DMH + celecoxib group: celecoxib was administered p.o. daily (ED_{50} for rats, 6 mg/kg body weight) to the

animals along with the weekly administration of 30 mg/kg body weight of DMH [10].

After 18 weeks, animals were kept on overnight fasting with drinking water ad libitum and killed under ether over anesthesia, the next day. The animal body weights in all the groups were recorded once in a week till the termination of the experiment.

Isolation of colonic epithelial cells

Colonic epithelial cells (colonocytes) were obtained from the freshly isolated colons by the method of Mouille et al. [13], as originally described by Roediger and Truelove [14]. The colonic segments starting from the cecum to the rectal ampulla were rapidly removed and flushed clear with chilled physiological saline (NaCl solution, 9 g/l) and then with Ca^{2+} and Mg^{2+}-free Krebs-Hanseleit (K–H) bicarbonate buffer (pH 7.4) containing 10 mM HEPES, 5 mM DL-dithiothreitol (DTT), and 2.5 g/l bovine serum albumin (BSA). The K–H buffer was equilibrated against a mixture of O_2 and CO_2 (19:1, v/v). Each colon was everted, distally ligated, and distended as much as possible by means of a syringe containing calcium-free K–H saline with 0.25% w/v BSA. These sacs were then placed in a plastic flask containing 100 ml calcium-free K–H buffer, 5 mM EDTA, and 0.25% BSA. The flask was gassed with O_2 and CO_2 (19:1 v/v) and incubated at 37°C in a shaking water bath at 60–70 oscillations per min for 30 min. Thereafter, the colons were rinsed in fresh calcium-free saline to remove the excess EDTA and placed in a plastic beaker with 50 ml K–H buffer and 0.25% w/v BSA. Manual stirring with a plastic stirrer for two min readily disaggregated the colonocytes, which were then separated by centrifugation at 500 g for 60 s. The cells were washed two times in oxygenated K–H buffer containing normal amounts of calcium (2.5 mM $CaCl_2$), 5 mM DTT, and 2.5% w/v BSA. The cells were washed and finally resuspended in PBS for further studies. Trypan Blue dye exclusion test was performed each time for every group of isolated colonocytes and the viability of cells was observed $\sim$90% (data not shown).

Western blot analysis

Protein samples/nuclear extracts (50 μg) from each group were separated on 10% SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose membrane (Millipore, Bangalore, India) and the transfer was checked by staining with Ponceau S. Immunoblot was prepared using primary antibodies (COX-2;1:1,000 and β-actin; 1:10,000) and alkaline phosphatase-conjugated IgG secondary antibodies (1:1000). BCIP–NBT detection system was used to develop the blot. Bands obtained were analyzed using Image J software (NIH, Bethesda, MD, USA) and the density expressed in gray values.

For preparation of cell lysates, colons were removed and rinsed from the different groups after completion of 18 weeks. Total lysates were prepared in fresh ice-cold lysis buffer (10 mM Tris, 100 mM NaCl, 5 mM EDTA, 1% Triton-X100, 1 mM PMSF, and 2 mM DTT (pH 8)). The extracts were cleared by centrifugation at 10,000$\times g$ for 10 min at 4°C and the supernatants were collected.

Immunofluorescence analysis

5 μm thick paraffin sections were deparaffinized in xylene for 30 min, gradually hydrated in descending series of alcohol (100, 90, 70, 50, and 30%) and finally brought to water. Sections were incubated at 95°C for 5 min in 50 mM Glycine–HCl buffer (pH 3.5) with 0.01% (w/v) EDTA for antigen retrieval, topped off with fresh buffer and again incubated at 95°C for 5 min. Slides were allowed to cool for approximately 20 min and washed in three changes of distilled water. The non-specific binding was blocked by incubating with 2% Bovine serum albumin (BSA) in phosphate buffered saline (PBS, 10 mM, pH 7.2) for 30 min at 37°C. The sections were then incubated in 1.5% BSA with primary antibody of COX-2 (1:500) in a moist chamber for 2 h at 37°C. For negative control only 1.5% BSA was added. After incubation, three washings were given with PBS, PBS Tween (PBS with 0.05% Tween 20) and PBS, successively, for 5 min each. The sections were then incubated with the FITC-conjugated secondary antibody at a dilution of 1:10,000 for 2 h at 37°C in dark. Sections were washed again in the same manner as described above, counterstained the sections with Propidium iodide for 20 min at 37°C in dark and washed as earlier. These were mounted in glycerol (1:10 in PBS), sealed with nail paint and observed under a fluorescence microscope at $\times$400.

Evaluation of membrane electric potential using merocyanine 540 (MC540)

Merocyanine 540 is a lipophilic fluorescent dye and has been associated with changes in membrane fluidity and electric potential. It is believed to stain cell membranes with increasing affinity as their lipid components become more disordered [15].

Isolated colonocytes (2×10^6 cells/ml) were labeled with MC540 (2.7 μM) in DMSO by incubating in dark for 15 min at 37°C [16]. Changes in membrane fluidity between different treatment groups were observed with a Carl Zeiss (Axioscope A1, Germany) fluorescence microscope at $\times$400. For quantification of cells, a total of 300

cells from four different slides were observed for each animal group.

Fluorescence measurements were carried out with a dual-wavelength Perkin Elmer Luminescence Spectrometer LS-55 (Beaconsfield, United Kingdom) using a 2 ml, 1 cm path length quartz cuvette installed with FL WinLab Software 4.00.02. Excitation and emission slits with a nominal band pass of 5 nm were used in the experiment. The cells (4×10^6 cells/2 ml) were loaded with MC540, by incubating for 15 min at 37°C in dark. Fluorescence from colonocyte cell suspensions was registered at emission wavelengths (λ_{em}) of 585 nm and excitation wavelength (λ_{ex}) of 488 nm.

Evaluation of lateral phase separation (*N*-NBD-PE fluorescence quenching)

Membrane lipid phase separations were monitored by the method of Nichols and Pagano [17] as described by Hoekstra [18]. The method is based on the self quenching of the *N*-NBD-PE fluorophore that occurs when the local concentration of NBD lipid in the bilayer increases during the segregation of the membrane lipids into the discrete domains. 2 ml vol containing 4×10^6 colonocytes were labeled with *N*-NBD-PE (final concentration 0.6 μM) by incubating in dark for 15 min at 37°C. The samples were loaded with *N*-NBD-PE using the same buffer (K–H buffer) as was used for the original isolation procedure. Fluorescence was assayed by measuring the intensities at $\lambda_{ex} = 475$ nm and $\lambda_{em} = 530$ nm before and after solubilization of the cells by the addition of SDS (100 μl, 1%) [17]. The percentage of quenched fluorescence ($\%Q$) was calculated from the relative fluorescence of unquenched *N*-NBD-PE (FU) in the presence of detergent and quenched molecules (FQ) by the relation: $\%Q = [(FU - FQ)/FU] \times 100$.

Evaluation of membrane phase state

Membrane phase state was determined to evaluate the polarity in terms of generalized polarization (GP) using fluorescent membrane probe, Laurdan (6-dodecanoyl-2-dimethylaminonaphtalene) following the method of Parasassi et al. [1] and as described by Ambrosini et al. [19]. The Laurdan molecule localizes at the hydrophobic-hydrophilic interface of the lipid bilayer (glycerol backbone) and its spectral properties are both due to the number and to the molecular dynamics of water dipoles in its environment and probably due to the effect of dipolar relaxation processes as well [4]. The environment-sensitive spectroscopic properties of Laurdan have been comprehensively described by a parameter termed GP, originally introduced by Gratton and co-workers [4]. In order to quantify the GP parameter the relative values of $exGP^{340}$ and $exGP^{410}$ (measured on excitation generalized polarization (exGP) at 340 and 410 nm) and $emGP^{440}$ and $emGP^{490}$ (measured on emGP at 440 and 490 nm) are considered important.

Calculation of GP parameter

2×10^6 cells/ml population of colonocytes were equilibrated for 20 min at 37°C in dark to be labeled with Laurdan (stock prepared in methanol) at 1.4 μM final conc. The buffer for colonocytes was kept the same as that of their original preparation for labeling the probe. Steady-state Laurdan excitation spectra were obtained in the range of 300–420 nm, using both 435 and 490 emission wavelengths. Similarly, Laurdan emission spectra were recorded in the range from 420 to 550 nm, using both 340 and 410 nm excitation wavelengths.

Blank spectra were obtained with unlabeled samples and subtracted from the spectra of the labeled samples. Readings were made at fixed temp of 37°C for all the samples. From the spectroscopic data, Laurdan exGP spectra were derived by calculating the GP value for each excitation wavelength [20]:

$$exGP = (I_{440} - I_{490})/(I_{440} + I_{490})$$

where I_{440} and I_{490} are the intensities measured at each excitation wavelength (from 320 to 420 nm), on the fluorescence excitation spectra obtained by fixed emission wavelength of 440 and 490 nm, respectively. Laurdan emission generalized polarization (emGP) spectra were similarly derived by calculating the GP value for each emission wavelength:

$$emGP = (I_{410} - I_{340})/(I_{410} + I_{340})$$

where I_{410} and I_{340} are the intensities measured at each emission wavelength (from 420 to 520 nm). The choice of 410, 340, 440, and 490 nm for GP calculation was based on the characteristic excitation and emission wavelengths of pure gel and liquid-crystalline lipid phases, according to Parasassi et al. [20].

Fluidity and order parameter assay using DPH

The fluorescent probe, DPH, was used in the membrane fluidity studies (rotational diffusion). DPH, a hydrophobic molecule, localizes and becomes fluorescent in the non-polar regions of the lamellar lipid aggregates. When excited by plane polarized light at 365 nm, DPH emits fluorescence that is polarized to an extent that depends on the viscosity of its localized molecular milieu, or microviscosity, i.e., in lipids with high microviscosity DPH fluorescence is highly polarized and vice versa [21]. A stock solution of 1 mM probe in

tetrahydrofuran (THF) was prepared and stored in a dark colored bottle being protected from light at room temperature. A small volume (10 μl) of DPH solution in THF was injected with rapid stirring into 1,000 volume of Tris/HCl buffer (10 ml) at room temperature. The suspension was stirred for at least 2 h after which little or no odor of THF was detected and the suspension showed negligible fluorescence. The colonocytes (2×10^6 cells/ml) were incubated in 2 ml of the above suspension containing 2 μl DPH for 2–4 h at 37°C in dark. Thereafter, estimations of fluorescence intensity (F), fluorescence polarization (P), and fluorescence anisotropy (r) were made with an excitation and emission wavelength of 365 and 430 nm, respectively. Order parameter (S) was calculated using the formula, $S^2 = (4/3r - 0.1)/r^o$ [19] taking r^o value for DPH as 0.362 and fluidity was measured in term of reciprocal of fluorescence polarization ($1/P$) [22].

Membrane microviscosity study using pyrene

Pyrene excimer (dimer) formation was used as a parameter of the lateral diffusion in the membrane [23]. The fluorescence of pyrene and many of its derivatives is a function of the microscopic concentration of the probe in the membrane. Pyrene has a highly structured fluorescence spectrum with wavelength maxima at less than 400 nm (397 nm) which is defined as the monomer emission (M). At higher concentration, a pyrene molecule in its first excited singlet state can, by a diffusion controlled process, associate with a ground state molecule to form an excited dimer or excimer (E) which fluoresces at a wavelength maximum of 472 nm. The ratio of these two intensities, E/M, is directly proportional to the pyrene concentration in the membrane hydrocarbon core [24] as defined by:

$$E/M = (\text{Excimer fluorescence}/\text{Monomer fluorescence}) = [\text{pyrene}]\, Tk/\eta$$

where T is absolute temperature, k is the Boltzmann constant (1.38062×10^{-23} J/K), and η is the viscosity.

Cells (2×10^6 cells/ml) were suspended in 2 ml of 0.25 M sucrose/1 mM EDTA (pH 7.0) and 2 μl of pyrene was added from a stock solution (5 μM) made in acetone and stirred for 1 h at 25°C. Final concentration of pyrene was set at 0.005 μM. Thereafter, excimer and monomer intensity were measured at excitation wavelength of 320 nm, and monomer emission (M) of 397 nm and excimer emission (E) of 472 nm.

Statistical analysis

Data were expressed as mean ± SD of four animals for each group. One way analysis of variance (ANOVA) was done to compare the means between the different treatments using post-hoc comparison by least significant difference method. The statistical software package SPSS v14 for windows was used for the purpose. A value of $P < 0.05$ was considered significant in this study.

Results

Macroscopic analysis of colon for tumor development

Everted colons were analyzed for tumor formation. Full grown tumors were observed in the case of DMH treated groups whereas the number and size of tumors was found to be less in NSAIDs co-administered groups (Fig. 1).

COX-2 expression studies

The expression level of COX-2 protein is higher in DMH alone group as compared to NSAIDs co-administered groups (Fig. 2a, b). DMH + sulindac group is having higher expression of COX-2 in comparison to DMH + celecoxib group suggesting that sulindac might follow COX-2 independent pathway of chemoprevention. Immunofluorescence study also reveals higher expression of COX-2 in colon sections of the DMH group in comparison to the Control and NSAIDs co-administered groups (Fig. 2c).

Membrane fluidity and potential analysis by merocyanine 540

Merocyanine 540 is a negatively charged heterocyclic chromophore having a permanently charged sulfonate group that restricts its membrane permeability thereby allowing the selective measurement of outer leaflet of plasma membrane. Binding and intensity of MC540 is affected by the membrane electric potential and packing of lipids in the bilayer. In DMH alone group we observed more of the electric potential and fluidity in plasma membrane of the isolated colonocytes (high intensity or brighter fluorescence) with respect to the Control group (Fig. 3a, b), which was reverted back by the administration of the two NSAIDs, sulindac, and celecoxib (lower intensity or dull fluorescence). These results were confirmed by spectrofluorimetric analysis of the samples as the DMH treated group is having highest intensity of MC540 with respect to Control and NSAIDs co-administered groups (Fig. 3c).

Membrane lateral phase separation analysis by self quenching of *N*-NBD-PE

NBD moiety possesses some of the most desirable properties to serve as an excellent probe for both spectroscopic

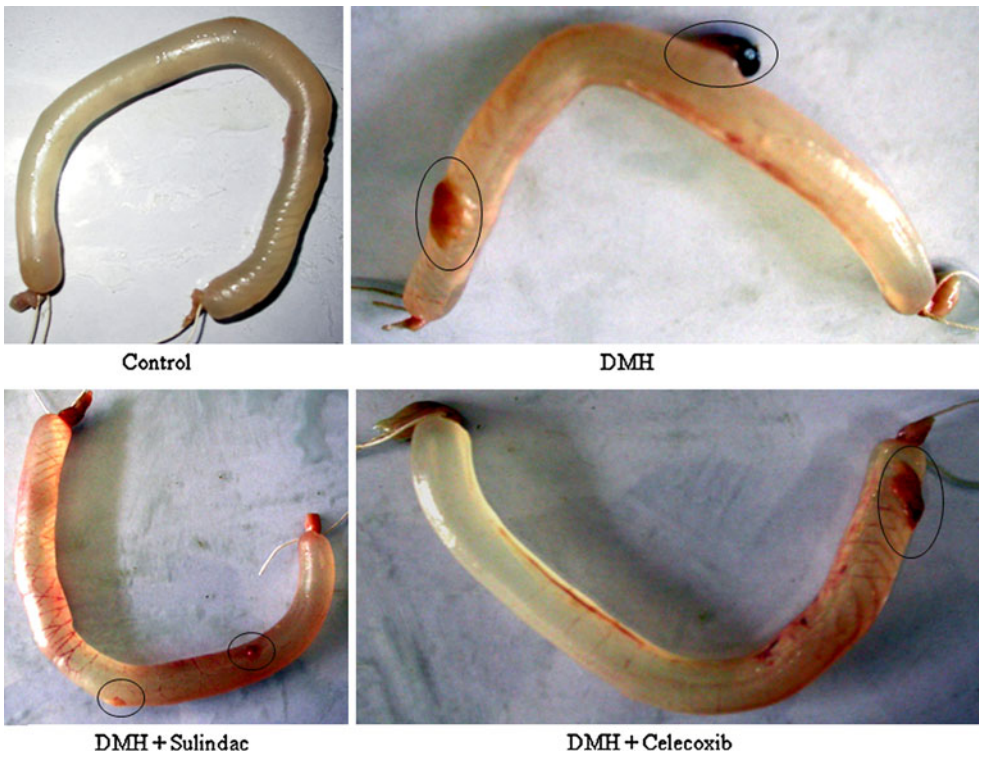

Fig. 1 Everted and ligated colon sacs from each group filled with calcium-free K–H saline with 0.25% w/v BSA showing tumor regions

and microscopic applications. It is very weakly fluorescent in water and upon transfer to a hydrophobic medium, fluoresces brightly in the visible range and also exhibits a high degree of environmental sensitivity. Separation of lipid phases leads to an increase in local concentrations of the NBD-lipid in the bilayer, and hence to self quenching. The rate and extent of NBD self quenching provides the measure of phase separation and thereby domain formation in the membrane. In Fig. 4, the colonocytes in DMH alone group show significantly less quenched fluorescence (*%Q*) with respect to the Control. NSAIDs co-administered groups, however, brought the *%Q* value significantly higher.

Evaluation of membrane phase state by Laurdan

To study the polarity of the lipid interfaces and to follow gel to liquid-crystalline phase transitions, Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) was used, which is an amphiphilic probe and can detect the coexisting domains. The excitation maximum in gel phase bilayer is at about 390 nm, while in the liquid-crystalline phase it is about 360 nm. Because of this difference in excitation, a partial photo-selection of Laurdan molecules surrounded by the gel phase with respect to those in the liquid-crystalline environment is possible. The environment-sensitive spectroscopic properties of Laurdan have been comprehensively described by a parameter termed GP. To quantify the changes, the relative values of $exGP^{340}$ and $exGP^{400}$ were measured on exGP spectra, and $emGP^{440}$ and $emGP^{490}$ were measured on emGP spectra (Fig. 5a, b). The values at $exGP^{340}$ and $exGP^{400}$ show that the gel phase transition is more in the NSAIDs co-administered groups with respect to DMH alone group as the values are more nearer to 0.6 which is said to be a well defined gel phase. On the other hand, the values at $emGP^{440}$ and $emGP^{490}$ represent the liquid-crystalline state which is more prominent in DMH alone group with respect to NSAIDs co-administered groups.

Fluidity and order parameter study of membrane

The steady-state fluorescence anisotropy of the colonocytes was assessed by DPH. Fluidity ($1/P$) or inverse of polarization and order parameter (S) were calculated according to the Perrin's equation (21). Figure 6a shows a significant decrease in fluidity or a shift toward gel phase in DMH + sulindac and DMH + celecoxib groups with respect to the Control and DMH alone groups.

Similarly, the order parameter (S) which represents the anisotropy of the membrane lipid state was observed significantly higher in case of DMH + sulindac and DMH + celecoxib groups with respect to the DMH alone group (Fig. 6b).

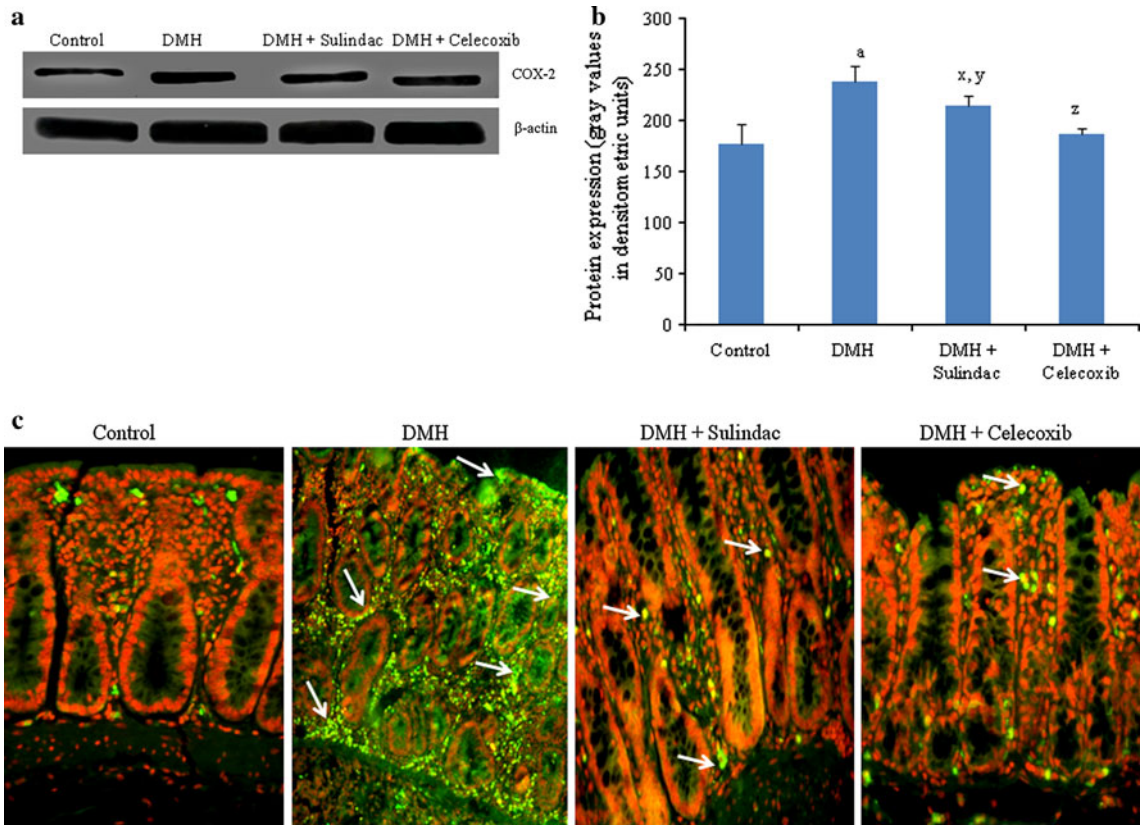

Fig. 2 **a** Western blots analysis of COX-2 and β-actin showing the effect of DMH and co-administered sulindac and celecoxib on the protein expression. DMH group is showing the higher expression of COX-2 protein whereas β-actin is serving as loading control (**b**) the densitometric analysis of the blots. The values are mean ± SD of three independent experiments with four different animals from each group. $^{a}P < 0.01$ in comparison to control and $^{x}P < 0.01$, $^{y}P < 0.05$, and $^{z}P < 0.01$ in comparison to DMH by one way ANOVA. **c** Immunofluorescence analysis of paraffin sections at ×400 representing the expression and localization of COX-2 expressing colonocytes (*arrows*) in different groups

Microviscosity analysis

Validation of steady-state anisotropy by DPH experiments was done using the concept of Pyrene Excimer formation and the microviscosity (η). Microviscosity is significantly less in the NSAIDs co-administered groups when compared with the Control and DMH alone groups (Fig. 7).

Discussion

The fundamental physicochemical properties of plasma membrane, e.g., electrostatic potential, fluidity, phase state, and polarization have strong effects on the biological functioning of the integral proteins or enzymes involved in many cellular process, such as proliferation, differentiation, malignancy, and programmed cell death or apoptosis [20]. Many spectroscopic techniques have been applied to characterize the structure and dynamics of phospholipids in plasma membrane. Fluorescence spectroscopy could be a sensitive technique for detecting these membrane properties [4].

According to Levine [25], NSAIDs might get intercalated into the plasma membrane of the cells and hence show the chemopreventive effect against carcinogenesis which could be via the alteration of signaling pathways. Earlier, we have shown that Diclofenac which is a preferential COX inhibitor has its role in the chemoprevention of early stages of experimentally induced colorectal cancer via reverting back some physicochemical changes essential for carcinogenesis [26]. In this study, we have investigated the chemopreventive effects of two NSAIDs, i.e., sulindac and celecoxib on the plasma membrane of isolated colonocytes, particularly in the late developmental phase of experimentally induced colorectal cancer by DMH which is a potent colon specific pro-carcinogen.

The expression and localization studies of COX-2 confirm that its higher expression might be responsible for the carcinogenesis induced by DMH. In comparison to celecoxib, sulindac at the present dosage used, seems to have a COX-2 independent mechanism of chemoprevention and the lower expression of COX-2 in that group could be due to some other indirect anti-inflammatory mechanism.

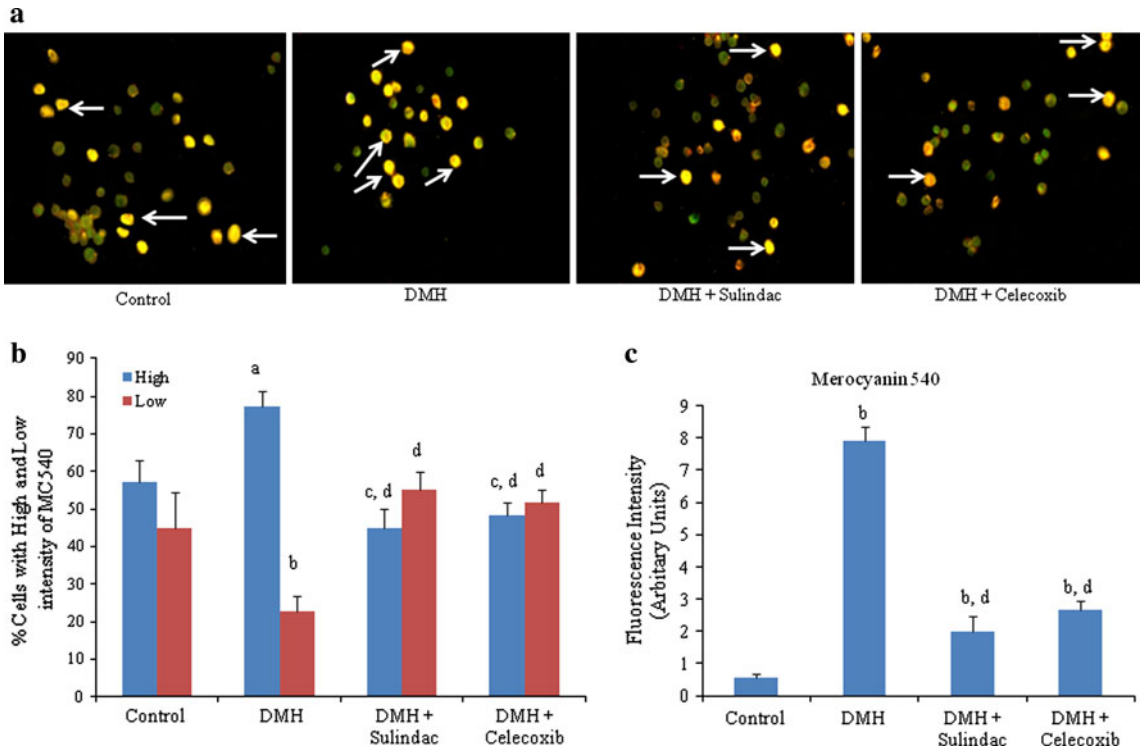

Fig. 3 **a** Fluorescent microscopic analysis (×400) of MC540 binding in the isolated colonocytes and the effects of DMH and co-administered sulindac and celecoxib on the MC540 fluorescence. High intensity or brighter fluorescent cells (*arrows*) are more in DMH group as compared to the Control and NSAIDs co-administered groups representing the higher electrical potential and more fluidity of plasma membrane in DMH treated animals. **b** Graph showing percentage of cells with high and low intensities of MC540 in the isolated colonocytes from each group counted under fluorescent microscope. **c** Spectrofluoremetric analysis of electric potential and fluidity of plasma membrane in isolated colonocytes labeled with MC540 and effects of DMH and co-administered NSAIDs on MC540 binding. The values are mean ± SD of four animals. $^{a}P < 0.01$, $^{b}P < 0.001$ in comparison to control and $^{c}P < 0.05$, $^{d}P < 0.001$ in comparison to DMH

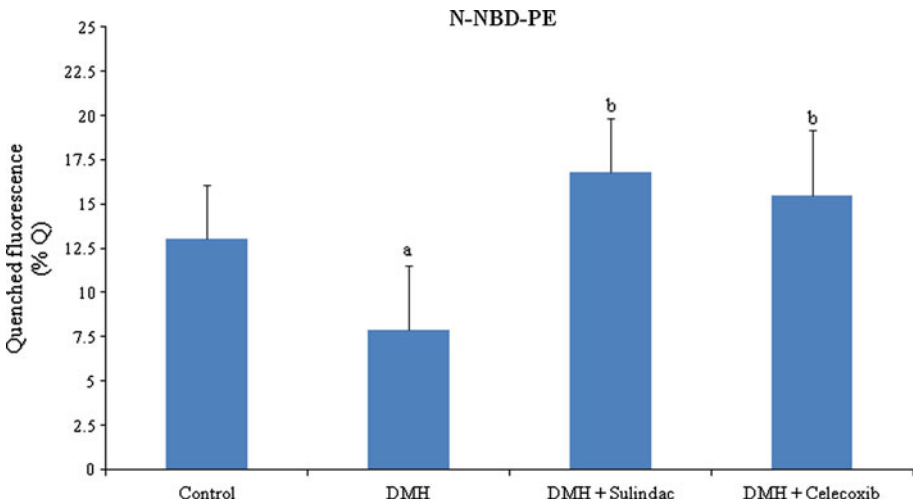

Fig. 4 Effect of DMH and co-administered sulindac and celecoxib on lateral phase separations in membranes of isolated colonocytes in terms of % fluorescence quenching (*%Q*), as measured by *N*-NBD-PE self quenching. The values are mean ± SD of four animals. $^{a}P < 0.05$ in comparison to control and $^{b}P < 0.01$ in comparison to DMH by one way ANOVA

Membrane electric potential was analyzed using a specific probe MC540 which is a negatively charged (sulfonate group) heterocyclic chromophore. MC540 is a lipid dye which especially stains distorted or loosely packed membranes, and also due to a permanently negative charge, the entry of MC540 is restricted to the outer leaflet of the lipid bilayers. Also, the membrane electric potential and phospholipids packing may strongly affect the binding efficiency. It has been reported that the leukemic cells bind MC540 with higher affinity in comparison to the normal circulating cells [27]. Due to such specific membrane binding properties of MC540, it is widely used in the

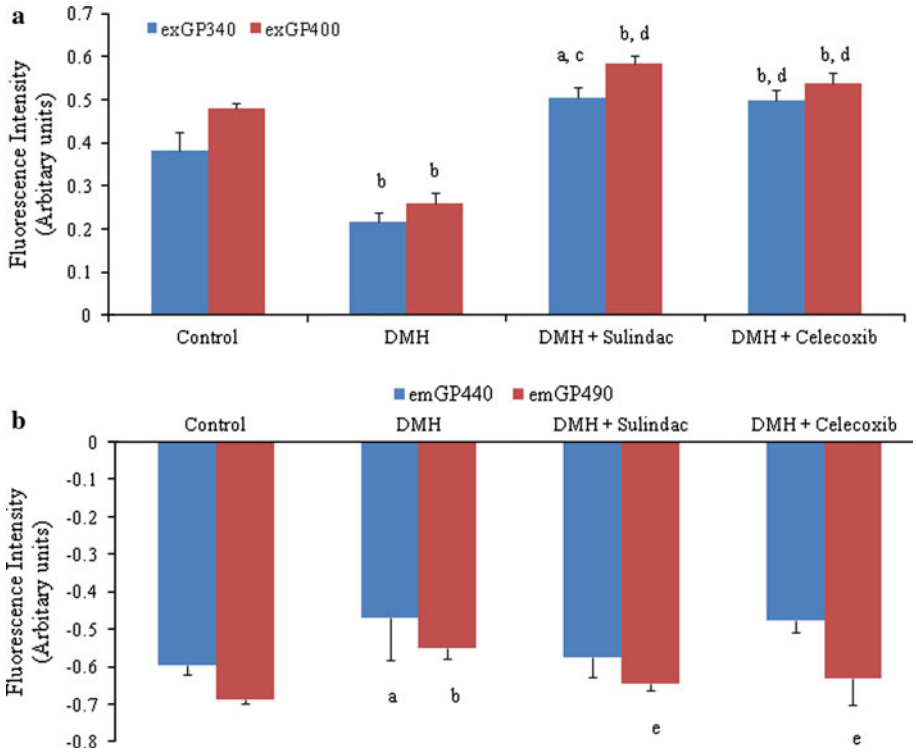

Fig. 5 Effect of DMH and co-administered sulindac and celecoxib on the Laurdan fluorescence excitation (ex) and emission (em) GP spectra in isolated colonocytes after blank correction. **a** Relative values of exGP340 and exGP400 as quantitated from the exGP spectra and **b** emGP440 and emGP490 as quantitated from the emGP spectra. The values are mean ± SD of four animals. $^{a}P < 0.05$, $^{b}P < 0.01$ in comparison to control; $^{c}P < 0.01$, $^{d}P < 0.001$, $^{e}P < 0.05$ in comparison to DMH; and $^{f}P < 0.001$, $^{g}P < 0.05$ in comparison to sulindac by one way ANOVA

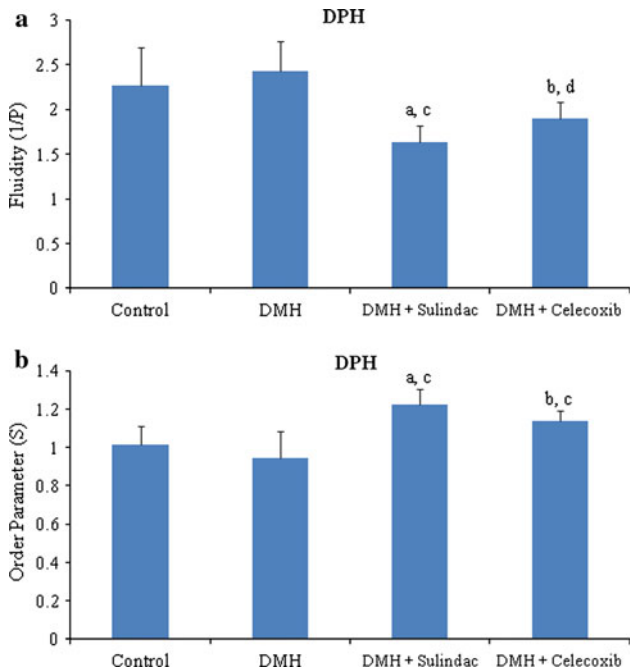

Fig. 6 Effect of DMH and co-administered sulindac and celecoxib on the membrane fluidity, $1/P$ (**a**) and order parameter, S (**b**) in isolated colonocytes as measured by DPH polarization. The values are mean ± SD of four animals. $^{a}P < 0.001$, $^{b}P < 0.05$ in comparison to control and $^{c}P < 0.001$, $^{d}P < 0.01$ in comparison to DMH by one way ANOVA

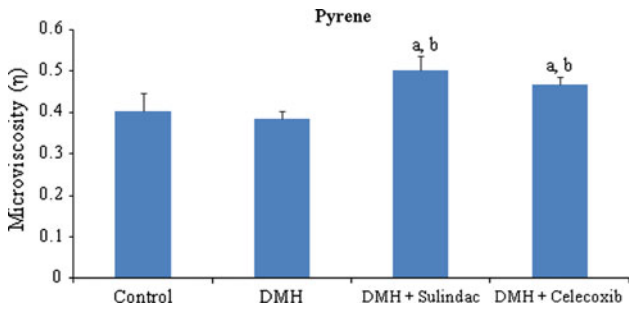

Fig. 7 Effect of DMH and co-administered sulindac and celecoxib on the membrane microviscosity (η) in isolated colonocytes as measured by E/M ratio of pyrene fluorescence. The values are mean ± SD of four animals. $^{a}P < 0.001$ in comparison to control and $^{b}P < 0.001$ in comparison to DMH by one way ANOVA

analysis of the metabolic conditions of both normal and cancer cells [28]. In this study, the higher membrane potential and fluidity of colonocytes in DMH group with respect to Control was noted by strong MC540 binding, while NSAIDs co-administered groups are having lower electric potential and fluidity.

To observe the modifications in translational movements (lateral) of the phospholipids in bilayers, *N*-NBD-PE was used which is a fluorescent probe whose self quenching occurs when these molecules are in close proximity with each other in the process of phase separation and resultant domain formation [29, 30]. Owing to higher fluidity or translational movement of phospholipids in plasma membrane of the colonocytes, DMH is having less $\%Q$ with respect to the Control group. NSAIDs co-administered groups have significantly higher value of $\%Q$ suggesting that colonocytes in NSAIDs co-administered groups are

having lesser fluidity of plasma membrane as compared to DMH one. It may be argued that NSAIDs may exert their chemopreventive effects against carcinogenesis via restricting the translational or lateral movements of phospholipids in the bilayers.

The phase state of membranes, i.e., either gels or liquid-crystalline, was studied using a fluorescent probe Laurdan which is an amphiphilic molecule synthesized by Dr. Weber [31] to study the dipolar relaxation process. A good shift in Laurdan emission spectrum toward longer wavelengths had been observed when examined for the dipolar relaxation process in high polarity solvents. In case of phospholipids a shift in Laurdan emission spectrum is dependent upon their composition and packing in bilayers as the molecules of Laurdan had been observed to be located in the hydrophilic-hydrophobic interface. For phospholipids, Laurdan GP values had been suggested to be valuable for the measurement of emission spectral shifts [32–35]. Laurdan in different phospholipid vesicles has a well defined value for the gel phase (GP $\sim$ 0.6) and for the liquid-crystalline phase (GP ~ -0.2) [4]. The value at $exGP^{340}$ in case of DMH group is ~ 0.2 which suggests that these colonocytes are having liquid-crystalline phase or more fluidity in their membranes while the values at $exGP^{400}$ of NSAIDs co-administered groups are nearer to 0.6 suggesting that these membranes are more in the gel state. The values of $emGP^{490}$ are more toward the gel phase suggesting that NSAIDs may revert back the carcinogenic changes as occurred by DMH administration in vivo.

The fluidity of the lipid bilayer can be described in terms of "microviscosity" which can be observed by the steady-state fluorescence polarization of DPH [36]. Fluorescence polarization was expressed as the fluorescence anisotropy (r), which is a probe dependent phenomena and reflect the overall motional freedom of the molecule. The steady-state anisotropy depends on two characteristics of probe: fluorescence lifetime and order parameter. The order parameter particularly is a static component of the membrane fluidity, and therefore important in the pathobiologic development of the cancer cells. The fluidity changes are calculated from the inverse of polarization values ($1/P$). It can be observed that during carcinogenesis induced by DMH for 18 weeks the cells are having more fluid plasma membrane with respect to Control. This suggests that membrane fluidity could be an important parameter for the study of carcinogenesis. However, significant decrease in fluidity was observed in NSAIDs co-administered groups with respect to DMH alone. This may attribute to the anti-neoplastic effects of NSAIDs via interaction with phospholipids in the bilayer.

Pyrene fluorescence examination is based on the lateral drift of the phospholipids in bilayers. If the membrane is more fluid then there should be more excited moieties (excimer) than the non-excited moieties (monomer) [37, 38]. The NSAIDs co-administered groups are showing significant decrease in E/M ratio with respect to DMH alone group stating that NSAIDs do have effect on phospholipids translational diffusions in bilayers and prevent excessive membrane fluidity. Microviscosity (η) of the membrane is inversely proportional to fluidity, i.e., more the fluidity less is the viscosity and vice versa.

Conclusion

This study utilizes the fluorescence spectroscopy techniques for the analysis of different fluidity parameters related to the plasma membrane of the isolated colonocytes in an experimentally induced colorectal cancer. Increase in plasma membrane fluidity in colonocytes might be related to the carcinogenesis in colon. Simultaneous administration of the NSAIDs with the pro-carcinogen, DMH, has shown the reverting effects of those agents toward normalizing the physical conditions in membrane and thereby offer a possible explanation as anti-neoplastic agents for their ability to prevent colorectal carcinogenesis in animal model.

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